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EFFECT OF DYNAMIC LOADING PARAMETERS ON THE BIOMECHANICS AND
BIOLOGY OF *IN VITRO* MODULATED GROWTH PLATE EXPLANTS

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EFFECT OF DYNAMIC LOADING PARAMETERS ON THE BIOMECHANICS AND
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DEDICATION

To my parents, for everything I have in my life...

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RÉSUMÉ

La croissance longitudinale des os est gouvernée par l'ossification endochondrale, un processus pouvant être affecté par les chargements mécaniques. La régulation de la croissance osseuse par l'environnement mécanique réfère à la modulation mécanique de la croissance. Ce processus est considéré comme un facteur clé de la progression des déformations angulaires pendant le développement, comme la scoliose juvénile et adolescente. Récemment, le concept de modulation mécanique a été exploité pour le développement des traitements sans fusion de la scoliose en modulant localement la croissance vertébrale. La croissance longitudinale des os s'opère au droit des plaques de croissance situées aux extrémités des os longs et des vertèbres. Elle résulte d'une séquence spatio-temporelle contrôlée des chondrocytes à travers les trois zones (réserve, proliférative, hypertrophique) de la plaque de croissance. Le processus commence par la prolifération des chondrocytes à la limite de la zone de réserve, puis les chondrocytes se divisent et prolifèrent rapidement en formant des colonnes verticales. Après prolifération, les chondrocytes s'hypertrophient; leur volume est augmenté jusqu'à 7 fois. Enfin, le cartilage se calcifie et est envahi par les vaisseaux sanguins. La forme, la taille et l'organisation des chondrocytes ainsi que la composition de la matrice extracellulaire sont différentes dans les trois zones de la plaque de croissance.

Dans cette étude, les effets des paramètres de chargement statiques et dynamiques sur les réponses biomécaniques et biologiques de la plaque de croissance ont été étudiés à trois niveaux. Tout d'abord, la viabilité des chondrocytes a été étudiée en réponse à différents paramètres. Cette étape a permis d'identifier l'ensemble des paramètres les moins dommageables pour la viabilité. D'autre part, les caractéristiques mécaniques des explants de la plaque de croissance ont été caractérisées en termes de patrons de déformation du tissu. Enfin, dans le but de trouver les relations entre les propriétés biomécaniques et les protéines structurales principales de la plaques de croissance, les contenus de protéines clés de la matrice extracellulaire ainsi que leur localisation ont été évalués.

Dans ce projet, des explants de plaques de croissance porcines de quatre semaines ont été utilisés. Les explants ont été divisés en dix groupes expérimentaux: 1) référence, 2) contrôle, 3) trois groupes statiques et cinq groupes dynamiques. La modulation mécanique a été effectuée en utilisant un bioréacteur sous des conditions standard de culture. Après modulation mécanique et

culture tissulaire ou immédiatement après la dissection pour les échantillons de type référence, chaque échantillon a été divisé en trois parties pour les trois niveaux différents d'études.

La première partie de chaque explant a été utilisée pour l'évaluation de la viabilité. Chaque partie a été colorée en utilisant la calcéine AM et l'homodimère d'éthidium 1 pour marquer les cellules vivantes et mortes, respectivement. Un algorithme de traitement d'images a été utilisé pour la quantification automatique du nombre de cellules vivantes et mortes. On a observé que la viabilité est dépendante de la magnitude, la durée, la fréquence, l'amplitude et la zone de la plaque de croissance. La zone hypertrophique a été identifiée comme celle la plus vulnérable. Aussi, pour une plus longue durée de chargement, la modulation statique a été plus dommageable pour la viabilité des chondrocytes que la modulation dynamique. L'augmentation de la fréquence et de l'amplitude a affecté la viabilité dans les zones proliférative et hypertrophique, respectivement. Basé sur les résultats de cette étude de viabilité, seulement les groupes qui ont montré une bonne viabilité ont été analysés pour les études subséquentes mécaniques et biologiques.

La deuxième partie du tissu a été utilisée pour la caractérisation mécanique et, par la suite, pour des analyses histomorphologiques de la plaque de croissance. La microscopie confocale, des tests de relaxation en contrainte et la corrélation d'images ont été combinés pour caractériser les patrons de déformation de chaque explant. Pour ce faire, les échantillons ont d'abord été marqués avec un fluorophore nucléique et placés entre les plateaux de chargement d'un appareil micro-mécanique installé sur l'objectif d'un microscope confocal inversé. Une première image a été sauvegardée avant d'appliquer le chargement puis une seconde image a été sauvegardée après application d'une déformation de 5%. Un algorithme de corrélation d'images a été utilisé pour évaluer les déplacements et les déformations entre les deux états. Les résultats ont montré que la modulation statique change le patron de déformation du tissu par rapport à la modulation dynamique. Par ailleurs, la modulation avec différents paramètres dynamiques ne modifie pas les patrons de déformation de la plaque de croissance. Suite aux tests mécaniques, les échantillons ont été enrobés dans du plastique puis colorés avec de la toluidine bleu pour des analyses histomorphométriques basées sur une méthode semi-automatique. Aucune différence significative n'a été observée entre les épaisseurs des trois zones de la plaque de croissance. Toutefois, dans le groupe statique, une perte de l'organisation colonnaire des chondrocytes a été observée dans les zones hypertrophique et proliférative. Dans les groupes dynamiques,

l'organisation cellulaire en colonne a été conservée mais avec des colonnes légèrement déviées par rapport à la direction de croissance.

Afin de trouver les relations entre les changements de propriétés biomécaniques et de protéines structurales, le contenu en protéoglycanes et collagène a été quantifié dans les trois zones de la plaque de croissance en utilisant des essais colorimétriques du bleu de diméthyl-méthylène (DMMB) et d'hydroxyproline. De plus, pour localiser précisément les protéines dans la matrice extracellulaire, l'aggrécane, et les collagènes de type II et X ont été marqués par immunohistochimie. Les intensités de réactions ont été quantifiées à l'aide d'une méthode de traitement d'images. Les courbes de relaxation de contrainte ont aussi été recalées par rapport à un modèle biphasique renforcé de fibres pour extraire le module d'élasticité de la matrice, le module des fibres et la perméabilité de chaque échantillon. Pour les échantillons modulés de façon statique, on a observé que l'augmentation de déformation du tissu était liée à l'augmentation de perméabilité, elle-même pouvant être liée à la perte de protéoglycanes dans les zones proliférative et hypertrophique des échantillons statiques. Le module de fibres et l'expression du collagène de type II n'ont pas été affectés par la modulation mécanique. Toutefois, le collagène de type X a été plus sensible au chargement mécanique et son expression a été réduite en réponse aux modulations statique et dynamique. Les modulations statique et dynamique avec amplitude élevée ont créé les réductions les plus importantes d'expression de collagène de type X.

Les première et troisième hypothèses de cette étude, indiquant que « *Les modulations statique et dynamique ont des effets différents sur la viabilité des chondrocytes, et que la modulation statique est plus dommageable* » et que « *Parmi les paramètres dynamiques de modulation, la fréquence (par rapport à l'amplitude de chargement) provoque les changements les plus importants sur les propriétés biomécaniques et la viabilité des chondrocytes* » ont ainsi été rejetées. La deuxième hypothèse de l'étude indiquant que « *Les explants chargés statiquement et dynamiquement montrent différents changements dans leurs propriétés biomécaniques et les explants dynamiques restent plus semblables aux échantillons référence* » a été confirmée. Enfin, la quatrième hypothèse indiquant que « *Des changements dans les propriétés biomécaniques et la viabilité des chondrocytes sont associés à des changements dans l'intégrité de la matrice extracellulaire et l'histomorphométrie tissulaire* » a été partiellement confirmée.

Cette étude est la première à investiguer les effets des paramètres de chargement statique et dynamique sur la viabilité cellulaire des plaques de croissance, leurs réponses biomécaniques et les relations avec leurs réponses biologiques. Dans l'ensemble, la modulation statique est plus dommageable pour les propriétés biomécaniques et la composition biologique de la plaque de croissance. En outre, pour une durée plus longue de chargement, le chargement statique a davantage de potentiel d'endommager la plaque de croissance comparativement au chargement dynamique. Cette étude fournit une meilleure compréhension de la modulation mécanique de la croissance, et des mécanismes impliqués dans la progression des déformations musculosquelettiques. À long terme, ces connaissances seront utiles pour le développement de nouvelles approches de traitement basées sur la modulation mécanique locale de la croissance osseuse.

ABSTRACT

Longitudinal bone growth is mediated through endochondral ossification, a process that can be affected by mechanical loading. The regulation of bone growth by mechanical loading is referred to as mechanical modulation of bone growth and is considered to be the underlying factor for the progression of angular developmental deformities, such as juvenile and adolescent scoliosis. Recently, the concept of mechanical modulation has been exploited for development of fusionless treatments of scoliosis by locally modulating vertebral bone growth. Longitudinal bone growth occurs in growth plate cartilage located at the extremities of long bones and vertebrae. Bone growth is the result of a precisely controlled sequence of cell proliferation and differentiation. The growth process starts with chondrocytes at the end of reserve zone of growth plate, which start to divide and proliferate rapidly in a vertical columnar arrangement. After their proliferation stage, chondrocytes undergo hypertrophy, where their volume is increased up to 7 folds. Finally, the cartilage is calcified and invaded by blood vessels. Not only the shape, size and arrangement of chondrocytes are different in the three growth plate zones but the composition of extracellular matrix is also different.

To date, many studies have evaluated the effects of different types of loading on growth plate responses with the purpose of finding the optimal loading condition for fusionless treatments. However still the effects of static vs. dynamic loading and dynamic loading parameters on biomechanical responses and biological responses of growth plate have not been fully identified. The goal of this thesis was to evaluate the effects of static vs. dynamic and dynamic loading parameters on growth plate biomechanical and biological responses. This has been investigated at three levels. First, chondrocytes viability was evaluated in response to each set of parameters. This step allowed identifying the most detrimental sets of parameters. Secondly, the mechanical characteristics of growth plate explants were characterized in terms of tissue strain patterns. Finally, in order to find the relationships between biomechanical properties and main structural proteins of growth plates, the protein content quantification and localization were performed.

Growth plate explants from 4-week old swine were used in this project. The explants were divided into ten experimental groups: 1) baseline, 2) control, 3) three static groups and five dynamic groups. Mechanical modulation was performed using a bioreactor maintained in standard culture conditions. After mechanical modulation and culturing or right after dissection

for baseline samples, each sample was divided into three parts for the three different levels of study.

The first part of each explant was used for viability assessment. Each part was stained using Calcein AM and Ethidium Homodimer 1 to label live and dead cells, respectively. An automatic image-processing algorithm was used for automatic quantification of the number of live and dead cells. It was observed that the viability was magnitude, duration, frequency, amplitude and zone dependent. The most vulnerable zone was found to be the hypertrophic zone. Also, for longer loading duration, static loading was found more detrimental for chondrocyte viability than dynamic loading. Increment of frequency and amplitude affected the viability of hypertrophic and proliferative zones, respectively. Based on the results of this viability study, only the mechanically modulated groups that showed good viability were further analyzed for their mechanical and biological responses.

The second part of the tissue was used for mechanical characterization and, thereafter, for histomorphological analyses. A combination of confocal microscopy, stress relaxation test and digital image correlation was used for mapping the strain patterns of each explant. First, the samples were labeled using a nucleic acid fluorophore and placed between the platens of a mechanical testing machine placed over the objective of a confocal microscope. A first image was recorded before applying any loading and, after applying a 5% strain and relaxation, a second image was recorded. Digital image correlation was further used to find the displacements and strain patterns between the two states. It was shown that static modulation changes the strain pattern of the tissue in comparison to dynamic modulation, while dynamic loading parameters did not affect growth plate tissue deformational patterns. After mechanical test, the samples were embedded in MMA and stained with Toluidine blue for histomorphometry using a semi-automatic method. No significant differences were found between the thicknesses of the three different zones of growth plate. However, in the static group a loss of columnar arrangement of chondrocytes was observed in hypertrophic and proliferative zones. In the dynamic groups, the columnar arrangements were preserved and the columns of cells were only deviated from the growth direction.

In order to find the relationships between changes in biomechanical properties and structural proteins, aggrecan and collagen contents of the three growth plate zones were quantified using

DMMB and Hydroxyproline colorimetric assays in the last part of the study. Moreover, to precisely locate the specific proteins, immunohistochemistry was used with aggrecan, type II collagen and type X collagen antibodies. The reaction signal intensities were quantified using a custom developed image analysis method. In addition, stress relaxation curves were fitted on fibril reinforced biphasic model and the matrix modulus, fibril modulus and permeability of each sample was evaluated. For statically modulated samples, it was established that the increased tissue strain after modulation is related to an increased permeability, which can be related to a loss of proteoglycan in the hypertrophic and proliferative zones of statically modulated samples. Fibril modulus and type II collagen expression was not affected by mechanical modulation. However, type X collagen was found to be the most sensitive extracellular protein to mechanical loading, where its expression was reduced in response to both static and dynamic modulation. Static modulation and dynamic modulation with high amplitude had the most detrimental effects on type X collagen expression.

The first and third hypotheses of this study, stating that: “*Static and dynamic modulation trigger different effects on chondrocyte viability, and static modulation will be more detrimental for cellular viability*” and “*Among dynamic modulating parameters, frequency causes the most significant changes in growth plate biomechanical properties and chondrocyte viability compared to loading magnitude*” were then rejected. The second hypotheses of the study stating that “*Statically and dynamically modulated growth plate explants show different changes in their biomechanical properties and dynamically modulated growth plate explants remain more similar to baseline samples*” was confirmed. Finally, the fourth hypotheses stating that “*Changes in growth plate biomechanical properties and chondrocyte viability are associated with changes in ECM integrity and tissue and cell histomorphometry*” was partly confirmed.

The main limits of this research project are associated with the *in vitro* approach using a single animal model at one site and at a single developmental stage on a healthy animal model. Moreover, only two variations of each parameter were tested. Also, at viability and mechanical characterization levels, only one test was used and the sample number was found not sufficient to provide conclusive results on protein studies.

Conversely, this study was the first to offer significant information on the effects of static and dynamic loading parameters on cellular viability, biomechanical responses and their relationships to the biological responses of growth plate tissue. Overall, static modulation was shown to be more detrimental for both tissue biomechanical properties and biological responses. Moreover, it was shown that, for longer loading duration, it has more potential for damaging the tissue compared to dynamic loading. This study provides an improved understanding of the fundamental changes in growth plate following mechanical modulation and of the mechanisms of abnormal growth progression in angular developmental deformities. In the long term, this knowledge will be useful for development of treatment approaches based on the local mechanical modulation of bone growth.

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LIST OF SYMBOLS AND ABBREVIATIONS

ECM	Extracellular matrix
MSC	Mesenchymal stem cells
PG	Proteoglycans
GAG	Glycosaminoglycans
COMP	Cartilage oligomeric matrix protein
MMP	Matrix Metalloproteinases
MMA	Methyl methacrylate
DMMB	Dimethylmethylene Blue Assay
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
IHC	Immunohistochemistry

INTRODUCTION

Longitudinal growth of long bones and vertebrae is a result of a very synchronized differentiation and proliferation of chondrocytes and of extracellular matrix secretion and formation of a cartilage template for bone formation (Farnum and Wilsman 1998, Stokes 2002, Ballock and O'Keefe 2003). This process is regulated by many factors including hormones, genetics, nutrition and mechanical environment. The later is referred to as the mechanical modulation of bone growth. Based on Hueter-Volkmann law, this mechanical modulation is the underlying principle for the progression of developmental growth abnormalities such as scoliosis. Based on this law, following the onset of deformity and the consequent unbalanced loading on the growth plate, bone growth is reduced in the bone region under higher compression and is increased on the less compressed region. As a result, the bone becomes wedged and, hence, the unbalanced loading is further increased and the deformity progresses even more.

Current treatments include bracing for mild cases of the deformity and surgical intervention combined with fusion of vertebrae for severe cases, which is not desirable since it limits the spinal movement, and should be performed after skeletal maturity. Recently, the concept of mechanical modulation has been exploited for fusionless correction of scoliosis by reversing the compressive loading on growth plate. Although some of these treatments have been previously used *in vivo* with animal models or clinical trials, the optimal parameters for the mechanical modulation of bone growth remain to be clearly determined. To date, many studies have investigated the effects of static loading on growth rate and growth plate histomorphology, which have all confirmed the Hueter-Volkmann law (Stokes 2002, Stokes, Gwadera et al. 2005, Guille, D'Andrea et al. 2007, Cancel, Grimard et al. 2009, Valteau, Grimard et al. 2011). Moreover, several studies have addressed *in vitro* or *in vivo* the effects of static vs. dynamic mechanical modulation on growth rate, growth plate histomorphological parameters and growth plate extracellular matrix protein expression (Cheng, Sutton et al. 2002, Sergerie, Parent et al. 2011, Valteau, Grimard et al. 2011, Ménard, Grimard et al. 2014). Based on these studies, both static and dynamic compressions are equally effective in reducing bone growth rate but dynamic loading seems to better preserve tissue histomorphology and protein expressions. However, the effects of static vs. dynamic modulation on the biomechanical responses and the relationships between changes in mechanical responses and protein expressions have not been addressed.

Moreover, the effects of dynamic loading parameters, such as frequency and amplitude, also remain to be studied. At the biomechanical level, growth plate mechanical properties have been characterized both at the bulk level and in each growth plate zone. Based on these studies, the biomechanical responses of growth plate can be described using biphasic model, and it has a zone dependent mechanical behaviour with its reserve zone two times more rigid than the proliferative and hypertrophic zones.

The main purpose of this thesis was to investigate *in vitro* the effects of static vs. dynamic compression and of dynamic parameters (frequency and amplitude) on growth plate biomechanical and biological responses. To do so, different techniques such as viability assay, confocal microscopy combined with fluorescent labeling, digital image correlation, histomorphology, immunohistochemistry and biochemical assays were used.

This thesis includes six chapters and is submitted as an article based thesis. The general organization of the thesis is as follows. Chapter 1 presents a literature review on the context of the research, the state of knowledge on mechanobiology of the growth plate and its mechanical properties, and methods used in mechanobiology studies of cartilaginous tissues. Chapter 2 introduces the rationale, hypotheses and objectives of the project. The body of this thesis is composed of three principal articles presented in Chapters 3 to 5. Chapter 3 presents the first article entitled: “*Compressive mechanical modulation alters the viability of growth plate chondrocytes in vitro*”, which was published in Journal of Orthopaedic Research. This article investigates the effects of compressive loading parameters on the viability of chondrocytes *in vitro*. Chapter 4 introduces the second article entitled: “*Growth plate cartilage shows different strain patterns in response to static vs. dynamic mechanical modulation*”. This article was published in Biomechanics and Modeling in Mechanobiology and introduces the responses of growth plate tissue to compressive mechanical modulation parameters. Chapter 5 presents the last article of this thesis entitled: “*Static modulation of growth plate explants reduces proteoglycan and collagen x expressions compared to dynamic modulation*”, submitted to the Journal of Orthopaedic Research. In this chapter the protein expressions and protein contents of the growth plate in response to mechanical compressive parameters are presented and their relationship to changes in mechanical responses are discussed. The last chapter discusses the overall results of the project and the connections between the articles and the reviewed literature. Finally, the contributions of this thesis are summarized and recommended future studies are presented.

CHAPTER 1 REVIEW OF THE LITERATURE

1.1 Bone growth and development

Bone development starts in the embryo via two distinct mechanisms: intramembranous and endochondral bone formation. Intramembranous formation, which is the mechanism for formation of most of craniofacial bones, is a result of direct mesenchymal condensations (Long and Ornitz 2013). Endochondral bones are formed through an intermediary cartilage. Most of mammalian skeletal system bones are formed through the endochondral bone development process (Boskey and Coleman 2010). In the following section, the formation of endochondral bones is explained in details.

1.1.1 Endochondral bone formation

Endochondral bone formation is the mechanism for development of most bones, including long bones and vertebrae in mammals. Figure 1-1 illustrates the process of endochondral ossification of long bones. The process starts by formation of a cartilage template, which is later transformed to bone. During embryonic development or postnatal growth, the cartilage template is formed by condensation of mesenchymal cells (Figure 1-1-A). This is followed by the differentiation of mesenchymal cells in the center of the condensations into chondrocytes. At this stage, the special extracellular matrix components of cartilage such as types II, IX, and XI collagen and proteoglycans, such as aggrecan, are secreted by the chondrocytes and the cartilage template is formed (Figure 1-1-B). The secretion of type I collagen is suppressed at the center, whereas cells located at the periphery secrete type I collagen and form the perichondrium. Later on, chondrocytes at the center of the cartilage proliferate and mature by undergoing hypertrophy (Figure 1-1-C). Finally, the center of the terminal hypertrophy zone is invaded by blood vessels, the cartilage is absorbed, a bone collar is deposited (Figure 1-1-D) and the primary ossification center is formed. A secondary center of ossification is formed during the first years of postnatal life by ossification of chondrocytes in the epiphyseal parts of the bone (Mackie, Ahmed et al. 2008, Long and Ornitz 2013).

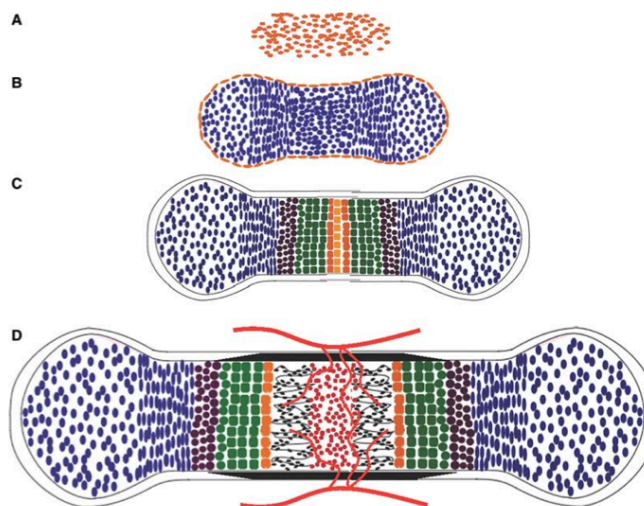


Figure 1-1 Endochondral bone formation, adapted from (Long and Ornitz 2013)

As the ossification centers grow, only two parts remain cartilaginous: (1) the part located at articular surface, which forms the articular cartilage (2) the part located between the epiphyses and metaphysis, which forms the growth plate. When the secondary ossification center is completely ossified, endochondral growth occurs only in the diaphyseal direction through the growth plate (Pansky 1982).

1.1.2 Growth plate

The remaining cartilages at the each end of the growing bones between the epiphysis and metaphysis are know as growth plates (Mackie, Ahmed et al. 2008) (Figure 1-2). Growth of long bones and vertebrae is a result of a precisely controlled differentiation and proliferation of growth plate chondrocytes. The growth plate can be divided into three distinct zones based on the stage of differentiation of its chondrocytes: reserve, proliferative and hypertrophic zones (Figure 1-3). The morphology of chondrocytes and biochemical composition of extracellular matrix is different in these three zones (Ballock and O'Keefe 2003, Mackie, Ahmed et al. 2008). Longitudinal bone growth is a very synchronized spatio-temporal differentiation and proliferation of chondrocytes throughout these three zones.

From prenatal stage until skeletal maturity (around the age of 20 years for human), longitudinal bone growth occurs in the growth plate. After skeletal maturity, the metaphyseal and epiphyseal bones are fused and the growth plate has disappeared (Mackie, Ahmed et al. 2008). However, in

some species, such as the rat, the growth plate remains present but becomes biologically inactive (Roach, Mehta et al. 2003).

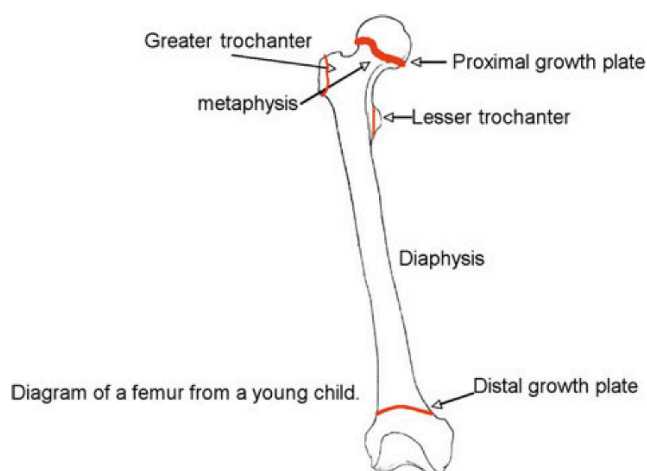


Figure 1-2 Anatomical positions of growth plates in a long bone (adapted from (Srinivas and Shapiro 2011))

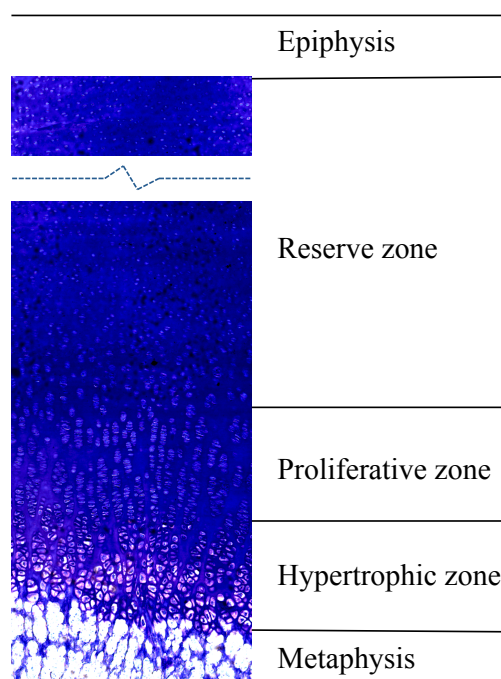


Figure 1-3 Representative histological section of a porcine growth plate showing the three zones of growth plate (toluidine blue staining).

1.1.2.1 Growth plate composition

1.1.2.1.1 Chondrocytes

Chondrocytes are differentiated from mesenchymal stem cells (MSCs) during embryonic development (Lin, Willers et al. 2006). They are the only cell type found in the growth plate and cartilage and are responsible for the synthesis and turnover of their extracellular matrix (Mow, Gu et al. 2005, Lin, Willers et al. 2006). In growth plates, there exist three different stages of differentiation: inactive in the reserve zone, proliferating in the proliferative zone and maturing in the hypertrophic zone. The chondrocyte shape, arrangement and size vary based on their state of differentiation (Ballock and O'Keefe 2003). After proliferation and hypertrophy, the chondrocytes are replaced by bone and undergo apoptosis, a programmed cell death (Álvarez, Balbín et al. 2000, Myllyharju 2014).

1.1.2.1.2 Extracellular matrix

Growth plate extracellular matrix is synthesized and secreted by chondrocytes. It is a structure of collagen fibrils embedded in a highly hydrated aggregating proteoglycan and hyaluronic acid complex (Álvarez, Balbín et al. 2000). The structure and concentration of these components differ in the three growth plate zones. In the following sections, each component is described in more details.

1.1.2.1.2.1 Collagen

Collagen is the main structural protein of the extracellular matrix in various mammalian connective tissues. In articular cartilage, collagen composes 10-20% of the wet weight the tissue (Mow, Gu et al. 2005). All collagens have a part consisting of three polypeptide chains (α -chains) coiled into a triple helix (Athanasίου, Shah et al. 2001).

Collagen has two major roles in cartilage and growth plate: (1) providing mechanical strength in tension (2) retaining the swelling pressure of the embedded proteoglycans and providing compressive strength (Mow, Gu et al. 2005). Collagen fibrils are formed by polymerization of collagen molecules. All collagens molecules are made of three polypeptide alpha chains coiled around each other in a triple helix configuration. The alpha chains can be identical or different, depending on the type of the collagen. Each polypeptide chain itself contains approximately 1000

amino acid residues (Mow, Gu et al. 2005, Shoulders and Raines 2009). Hydroxyproline and hydroxylysine are two characteristic amino acids of collagen, which are not found in other proteins (Shoulders and Raines 2009). Variation in the assembly of polypeptide alpha chains, length of helix, and different interruptions in the helix result in different type of collagens, which can be categorized into 3 groups: (1) fibril-forming, (2) network-forming and (3) fibril-associated collagens (Mow, Gu et al. 2005, Shoulders and Raines 2009). The major collagen present in growth plate ECM is type II collagen. Other characteristic collagens of the growth plate are types IX, X, and XI (Álvarez, Balbín et al. 2000, Myllyharju 2014).

- Type II collagen is a fibril-forming collagen composed of three identical $\alpha 1(\text{II})$ collagen chains. It has a role in providing the tensile strength to the tissue (Ballock and O'Keefe 2003, Mow, Gu et al. 2005, Myllyharju 2014).
- Type IX collagen is a fibril-associated collagen with three different collagen chains $\alpha 1(\text{IX})\alpha 2(\text{IX})\alpha 3(\text{IX})$ to which glycosaminoglycan components are linked to. This type of collagen attaches to type II collagen and mediates its binding to other ECM components (Balmain, Leguellec et al. 1995, Ballock and O'Keefe 2003, Myllyharju 2014).
- Type X collagen is a network-forming collagen and is exclusively found in the hypertrophic zone of growth plate. It is composed of three identical polypeptide alpha chains $\alpha 1(\text{X})_3$. It has a role in conversion of hypertrophic cartilage to bone (Keene, Oxford et al. 1995, Myllyharju 2014).
- Type XI collagen is a fibril-forming collagen; it has a role in regulating the size of type II collagen and also in mediating its interaction with proteoglycans (Ballock and O'Keefe 2003).

1.1.2.1.2.2 Proteoglycan

Proteoglycans are another major extracellular matrix component in connective tissues. In articular cartilage, PGs compose 5-10% of the wet weight of the tissue (Mow, Gu et al. 2005). Proteoglycans are composed of a main core protein to which one or several GAG chains are covalently attached. GAGs are the carbohydrate parts of proteoglycans, consisting of disaccharide subunits (Figure 1-4). In cartilage and growth plate, PGs normally exist in

aggregates formed by binding of the PGs monomer to hyaluronic acid, via link proteins (Figure 1-4 b) (Poole, Pidoux et al. 1982).

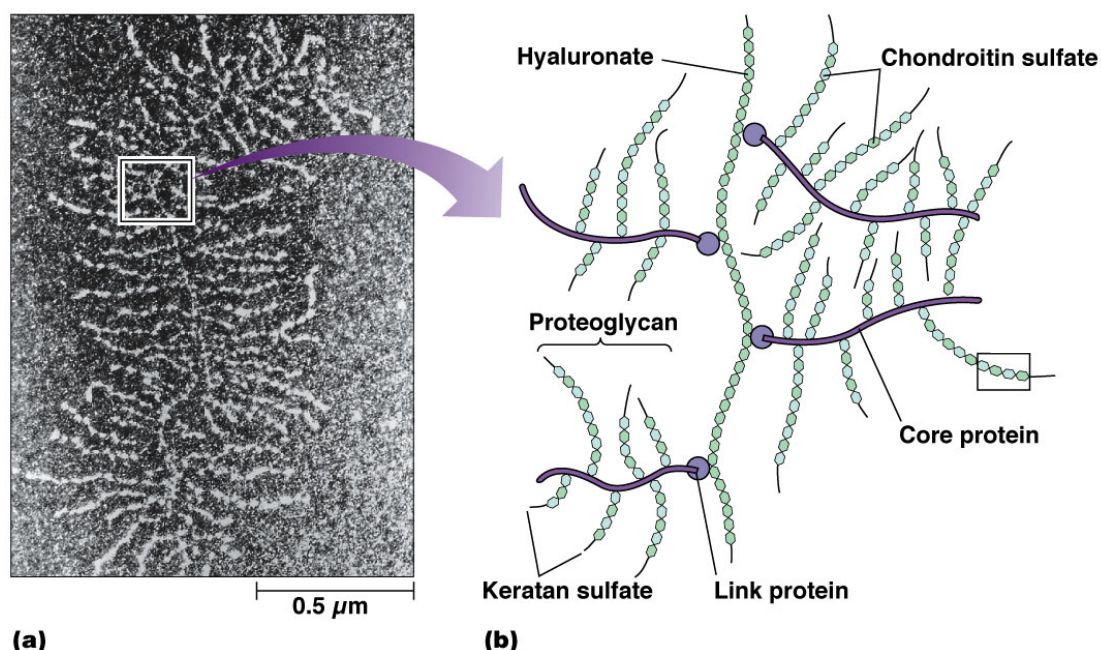


Figure 1-4 Proteoglycan structure (Adapted from 2012 Pearson Education, Inc)

The function of each proteoglycan is determined by its core protein and its GAG chains. proteoglycans have two major roles in the growth plate: (1) providing mechanical strength in compression (2) providing a scaffold to maintain cellular organization of chondrocytes necessary for normal growth (Roughley 2006). Moreover, it is also suggested that PGs have a role in matrix calcification in the hypertrophic zone (Poole, Pidoux et al. 1982).

The characteristic PG in the growth plate is aggrecan. Other PGs include perlecan, decorin, fibromodulin and lumican (Myllyharju 2014). The most abundant GAG in articular cartilage and growth plate is chondroitin sulphate, which is covalently attached to aggrecan (Knudson and Knudson 2001).

- Aggrecan is a large chondroitin sulfate PG with a core protein with molecular weight of ~230 kDa (Álvarez, Balbín et al. 2000, Myllyharju 2014). The chondroitin sulfate chains of aggrecan create a high charge density, which provides the osmotic properties for resisting compressive forces (Mow, Gu et al. 2005, Roughley 2006).

- Perlecan is a heparan sulfate/chondroitin sulfate PG and it has a role in mechanical strength of pericellular matrix of articular cartilage (Wilusz, DeFrate et al. 2012).
- Decorin has one chondroitin or dermatan sulfate side chain and is associated with collagen fibrils as a decorating PG (Knudson and Knudson 2001).
- Fibromodulin can have up to four keratan sulfate side chains and it has a role in regulating collagen fibril diameter (Knudson and Knudson 2001).
- Lumican is a keratan sulfate PG that binds to collagen during fibril formation and decreases the final diameter of the collagen fibrils (Poole, Kojima et al. 2001).

1.1.2.1.2.3 Water

Interstitial water is the most abundant component of ECM in cartilage and growth plate. It composes 68-85% of articular cartilage and 79-83% of growth plate ECM (Mow, Gu et al. 2005, Amini, Mortazavi et al. 2013). In articular cartilage, almost 30% of water resides within the intrafibrillar space between the collagen and a small portion of water resides in intracellular space. The rest of water resides in the porous ECM (Athanasίου, Shah et al. 2001). The proteoglycan content of the tissue and the organization of collagen fibers affect the water content of the cartilage and growth plate (Mow, Gu et al. 2005). Water is responsible for gas and nutrition transfer between chondrocytes and ECM. Also, all the inorganic ions such as sodium, calcium, chloride, and potassium are dissolved in water (Athanasίου, Shah et al. 2001). Under compression, water is exuded from the ECM and the frictional drag resistance of water with extracellular matrix creates the viscoelastic mechanical behaviour of the tissue under compression (Athanasίου, Shah et al. 2001, Mow, Gu et al. 2005).

1.1.2.1.2.4 Other noncollagenous proteins

- COMP is a large pentameric glycoprotein, which has a role in binding other ECM proteins, catalyzing polymerization of type II collagen fibrils, activating other MMPs and regulating chondrocyte proliferation (Posey and Hecht 2008).
- Matrilins are multi-domain proteins that associate with collagen fibrils and bind covalently to aggrecan and thus act as ECM assembly components (Myllyharju 2014).

- Tenascin-C is a large macromolecule found in the ECM of growth plate and cartilage, it might have a role in regulating the multiplication of chondrocytes in growth plate (Sandell, Heinegard et al. 2007).

1.1.2.1.2.5 Matrix Metalloproteinases (MMPs)

MMPs are proteolytic enzymes present in growth plate and cartilage. They have a variety of roles in chondrocyte proliferation, ECM protein degradation and release of their products, regulation of MMP and other proteinase activities, differentiation and programmed cell death (Malemud 2005). MMPs can be categorized in four main groups: collagenases, gelatinases, stromelysins and membrane type MMPs (Takahashi, Onodera et al. 2005). In growth plate, MMP-13, 9, 3 and 2 are present (Boskey, Spevak et al. 2002).

- MMP-13 or Collagenase-3 is the main protease involved in degradation of type II and type X collagens, aggrecan and other proteoglycans. It is exclusively secreted by hypertrophic chondrocytes (Mackie, Ahmed et al. 2008).
- MMP-9 or Gelatinase-B only cleaves denatured collagens and aggrecan (Mackie, Ahmed et al. 2008). Also, it might have a role in controlling the angiogenesis (Vu, Shipley et al. 1998).
- MMP-3 or stromelysin-1 affects PG, fibronectin, laminin and latent collagenase (Srinivas and Shapiro 2011).
- MMP-2 is a gelatinase and has a role in activation of MMP-13 pro-enzyme (Srinivas and Shapiro 2011).

1.1.2.1.2.6 Aggrecanases

Aggrecanases are a family of a disintegrin and metalloproteinase with thrombospondin motifs (ADAMT) proteolytic enzymes. ADAMTS-4 (aggrecanase-1) and ADAMTS-5 (aggrecanase-2) are the two aggrecanases present in growth plate. They are responsible for degradation of aggrecan and other proteoglycans (Mitani, Takahashi et al. 2006).

1.1.2.2 Growth plate Mechanical characteristics

The mechanical behavior of growth plate is inhomogeneous and anisotropic in both compression tension, because of variation in collagen and proteoglycan contents and random orientation of collagen fibers (Mow, Gu et al. 2005) along the growth plate thickness.

Similar to other cartilaginous tissues, the mechanical response of growth plate in compression is best described by a biphasic (or poroelastic) model. In this model, the growth plate is considered as a material with two major phases, a solid phase consisting of ECM and chondrocytes and a fluid phase composed of water and ions. The specific mechanical characteristics of growth plate result from the interaction of these two phases. The viscoelastic response under compression is a result of frictional drag of the fluid phase through the solid phase. (Mow, Gu et al. 2005). The mechanical characteristics of growth plate in compression have been extracted for several animal models in different studies. Unconfined stress-relaxation test results were fitted using a non-linear biphasic (Cohen, Chorney et al. 1994), transversely isotropic biphasic (Cohen, Lai et al. 1998, Sergerie, Lacoursiere et al. 2009, Wosu, Sergerie et al. 2012) or fibril-network reinforced biphasic (Ménard, Soullisse et al. 2014) models. Table 1-1 presents a summary of the mechanical properties of growth plate in compression.

Since mechanical properties of the growth plate vary in different zones, several study have investigated the zone based mechanical responses in compression. Using digital image correlation of confocal images taken during stress relaxation test (Villemure, Cloutier et al. 2007, Amini, Mortazavi et al. 2013), it was shown that for big animal models, such as the swine, deformation is higher in the proliferative zone while for smaller animal models, it is higher in the reserve zone. These results have been confirmed by another *in vitro* study, measuring the mechanical characteristics of the three zones of growth plate separately (Sergerie, Lacoursiere et al. 2009). In this study, the elastic modulus of the reserve zone was found to be two times larger than the two other zones.

Table 1-1 Mechanical characteristics of growth plate from unconfined compression stress relaxation studies. E_3 and E_1 are the elasticity modulus in the longitudinal and transversal directions, respectively. ν_{31} and ν_{21} are the Poisson ratios in longitudinal and transversal directions (parameters are calculated using a transversely isotropic biphasic model (Cohen, Lai et al. 1998)).

K_1 is the permeability in the longitudinal direction. E_m and E_f are the matrix and fibril elasticity modulus and σ_{max} and σ_{eq} are the maximum and equilibrium stresses (parameters are calculated using a fibril reinforced biphasic model (Korhonen, Laasanen et al. 2003)).

Study	Animal model	E_3 (MPa)	E_1 (MPa)	v_{21}	v_{31}	K_1 ($\times 10E-15 m^4/N.S$)
Wosu et al. 2012	4 weeks swine	0.95 \pm 0.14	9.74 \pm 2.11	0.28 \pm 0.08	0.066 \pm 0.0	2.04 \pm 0.61
	A				19	
	4 weeks swine	0.57 \pm 0.09	0.41 \pm 0.07	0.38 \pm 0.08	0.07 \pm 0.00	20.9 \pm 7.62
	B				2	
Sergerie et al. 2009	New born swine	0.51 \pm 0.12	8.65 \pm 1.72	0.24 \pm 0.07	0.08 \pm 0.03	1.82 \pm 0.67
Cohen et al. 1998	4 month bovine	0.47 \pm 0.11	4.55 \pm 1.21	0.30 \pm 0.20	0.0	5.0 \pm 1.8

Study	Animal model	E_m (MPa)	E_f (MPa)	σ_{max} (MPa)	σ_{eq} (MPa)	K_1 ($\times 10E-15 m^4/N.S$)
Menard et al. 2014	4 week swine	2.16 \pm 0.56	29.46 \pm 1.32	0.39 \pm 0.05	0.11 \pm 0.03	1.02 \pm 0.22

In tension, growth plate was found to be viscoelastic as the tangent modulus and ultimate stress are strain rate dependent (Williams, Do et al. 2001).

1.1.2.3 Growth plate zones

As already mentioned in section 1.1.2, based on the stage of differentiation of the chondrocytes, growth plate can be divided into three distinct zones: reserve, proliferative and hypertrophic

zones (Myllyharju 2014). Each zone has specific biochemical composition and mechanical response. In the following sections, the characteristics of each zone are described in more details.

Table 1-2 Mechanical characteristics of growth plate in tension calculated from stress-strain curve. $\dot{\epsilon}$ is strain rate, σ_U and ϵ_U are ultimate stress and strain respectively and E_t is tangent modulus.

Study	Animal model	$\dot{\epsilon}$ (s^{-1})	σ_U (MPa)	ϵ_U (%)	E_t (MPa)
Williams et al. 2001	5 week bovine	0.008	0.65±0.12	23±6	7.52±2.15
	12-18 week bovine	0.008	0.50±0.15	38±13	6.89±1.96

1.1.2.3.1 Reserve zone

The reserve zone, which is also known as the resting zone, is adjacent to the epiphysis. The chondrocytes in this zone have a spherical shape and are irregularly scattered throughout an ECM sometimes single or sometimes in pairs (Hunziker 1994). The highest concentration of type II collagen can be found in this zone (Abad, Meyers et al. 2002, Amini, Mortazavi et al. 2013). The collagen fibers do not have a specific orientation in this zone but most of them are aligned horizontally in a radial direction (Abad, Meyers et al. 2002, Amini, Mortazavi et al. 2013). The ratio of ECM to cell volume is high in this zone (Abad, Meyers et al. 2002, Ballock and O'Keefe 2003).

The reserve zone has several major functions in the growth plate: (1) production of ECM; (2) reservoir for stem like cells that are eventually differentiated to proliferative cells; (3) secretion of growth plate orienting (GPOF) morphogen, which organizes the proliferating and hypertrophic cells along the growth direction; (4) secretion of growth plate hypertrophy inhibiting morphogen, which prevents premature hypertrophy of proliferative cells (Abad, Meyers et al. 2002).

Based on biomechanical analyses in large animal models, this zone has the highest rigidity (Sergerie, Lacoursiere et al. 2009, Amini, Mortazavi et al. 2013) and serves as a structural support for longitudinal growth (Farnum and Wilsman 1998). Moreover, this zone has the

potential to regenerate the two other zones, which emphasize the underlying importance of this zone in growth plate (Abad, Meyers et al. 2002).

1.1.2.3.2 Proliferative zone

In the proliferative zone, chondrocytes proliferate and divide rapidly as their shapes become flattened and ellipsoidal. They are arranged in longitudinal multicellular clusters (Farnum and Wilsman 1998, Ballock and O'Keefe 2003). In this zone, chondrocytes secrete an ECM high in type II collagen, type IX collagen and proteoglycans (Noonan, Hunziker et al. 1998, Wongdee, Krishnamra et al. 2012). In early proliferative zone, collagen fibers are arranged in the longitudinal direction (Noonan, Hunziker et al. 1998, Amini, Mortazavi et al. 2013). Also, degrading enzymes such as MMP-2, 9 and 13 are secreted by the chondrocytes of this zone (Álvarez, Balbín et al. 2000, Takahashi, Onodera et al. 2005). In the late proliferative zone, cells start to enlarge and the proteoglycans and alkaline phosphatase concentration increase (Noonan, Hunziker et al. 1998).

The main role of this zone is maintaining chondrocytes proliferation, which directly affect bone growth rate. The proliferation rate of chondrocytes in this zone is correlated with growth rate of the bones and it changes with developmental stages. Therefore, from a histomorphological point of view, the number of chondrocytes in the columns and the thickness of this zone also vary with developmental stages and are reduced when approaching growth maturity (Farnum and Wilsman 1998).

1.1.2.3.2.1 Hypertrophic zone

In the hypertrophic zone, chondrocytes mature and their volumes are increased up to 10 folds. As a result, this zone has the lowest ECM to cell ratio (Noonan, Hunziker et al. 1998). In the late hypertrophic zone, type X collagen is expressed (Noonan, Hunziker et al. 1998, Mwale, Tchetina et al. 2002), the expression of type II collagen is decreased (Mwale, Tchetina et al. 2002), the size of proteoglycan aggregates is also increased (Noonan, Hunziker et al. 1998) and the interterritorial ECM starts to calcify (Noonan, Hunziker et al. 1998). MMP-13 expression is high in this zone (Mwale, Tchetina et al. 2002) and ADAMTS-4 and 5 are mainly expressed by mature hypertrophic chondrocytes (Mitani, Takahashi et al. 2006).

This zone has a role in chondrocyte maturation and hypertrophy, which directly correlate with bone growth rate. From a histomorphological point of view, the thickness of this zone and height of hypertrophic chondrocytes vary with growth rate (Wilsman, Leiferman et al. 1996, Farnum and Wilsman 1998). Because of changes in the ECM of this zone, this zone has the lowest stiffness among the three zones (Sergerie, Lacoursiere et al. 2009, Amini, Mortazavi et al. 2013).

1.1.3 Longitudinal growth postnatal until skeletal maturity

Postnatal longitudinal growth occurs within growth plates. The process starts with reserve chondrocytes, which secrete ECM proteins such as type II collagen and proteoglycans. At the end of this zone, they start to proliferate and push the older cells toward the diaphysis (Wongdee, Krishnamra et al. 2012). The proliferating chondrocytes become flattened and gathered in multicellular clusters as they progress (Mackie, Ahmed et al. 2008) and they secrete more type II collagen and proteoglycans. At the end of proliferative zone, chondrocytes start to increase their volume and they undergo hypertrophy. At the end of this zone, the hypertrophic chondrocytes start to secrete type X collagen. Finally, mature chondrocytes undergo apoptosis and the growth plate cartilage is invaded by blood vessels, osteoblasts, and hematopoietic cells. Matrix calcification occurs at this step, with the growth plate acting as a scaffold for the formation of new bone and deposition of hydroxyapatite. (Wongdee, Krishnamra et al. 2012)

1.2 Factors controlling longitudinal growth

In order to have normal bone growth resulting in proportioned limbs and perfectly angled limbs, the longitudinal growth of bones must be precisely controlled (Ballock and O'Keefe 2003). Many factors, including systemic regulation, local regulation, nutrition, genetics and mechanical environment regulate bone growth (Trueta and Morgan 1960, Van der Eerden, Karperien et al. 2003, Mackie, Ahmed et al. 2008, Myllyharju 2014).

- Systemic regulation

The major systemic hormones regulating growth during childhood include growth hormone (GH), insulin-like growth factor I (IGF-I), thyroid hormones (T3 and T4) and glucocorticoids while during puberty the sex steroids (androgenic steroids and estrogen) mainly regulate growth (Van der Eerden, Karperien et al. 2003).

- Local regulation

The most important locally acting growth factors that regulate bone growth include: Indian hedgehog (Ihh), PTH-related peptide (PTHrP), fibroblast growth factors (FGFs), bone morphogenetic proteins (BMPs), and vascular endothelial growth factor (VEGF) (Van der Eerden, Karperien et al. 2003).

- Nutrition

Nutrition is another parameter that regulates growth of bones, suppression of blood supply first damage the cells in the reserve zone, which eventually perturb the proliferation of cells in the hypertrophic zone and maturation in the hypertrophic zones and finally results in disintegration of growth plate (Trueta and Morgan 1960) .

- Genetics

It has now been widely accepted that genetic factors may be involved in some aspects of scoliosis including the curve shapes and the potential for curve progression (Good 2009). Several studies have documented that scoliosis runs within families and that the prevalence of scoliosis is higher among relatives of patients with scoliosis (Thomas, Michael et al. 1996).

1.2.1 Mechanical modulation of bone growth

In addition to the factors mentioned in the previous section, the mechanical environment of growth plate can regulate growth rate. Based on many experimental studies, increased compression on growth plate reduces growth rate and decreased compression increases growth rate (Stokes 2002). This phenomenon known as Hueter -Volkmann principle is mainly important in the progression of developmental angular deformities.

1.2.2 Abnormal growth and developmental growth angular deformities

Mechanical modulation of bone growth is believed to be the underlying reason for progression of angular deformities during growth. Upon the onset of the deformity and creation of bone wedging, the growth plate will undergo asymmetric loading. Higher compression will be applied to the concave side of the curve and lower compression to the convex side. As a result, the concave side will even have a slower growth rate and the convex side a faster growth rate, which

then results in the progression of the deformity within a vicious cycle. (Stokes, Spence et al. 1996)

1.2.2.1 Musculoskeletal pathologies involving longitudinal bone growth and their treatment

1.2.2.1.1 *Tibia vara*

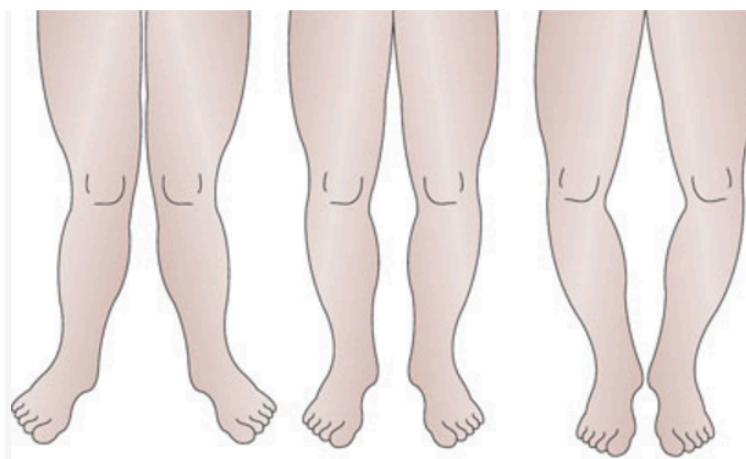
Tibia vara or Blount's disease is the developmental angulation of tibia into a varus deformity at its proximal end and internal tibial torsion (Langenskiöld 1989). The etiology is not very well understood but its progression results from the mechanical modulation of growth plate (Meier and Falls 2015). Treatment options include bracing for moderate cases, hemiepiphysiodesis 1-2 year before growth cessation for moderate cases or surgical intervention for aligning the tibia for severe cases (Meier and Falls 2015).

1.2.2.1.2 *Genu varum / valgum*

Both Genu varum and Genu valgum are believed to occur as a result of unbalanced loading on growth plate. Genu varum is a deformity marked by medial angulation of the leg from the thigh, giving the appearance of an arc. Genu valgum, or a "knock kneed", is marked by a deviation of lower limb to outside to the outside in a way that the knees touch while the ankles are separated (Figure 1-6).



Figure 1-5 Tibia vara (Adapted from (Center 2003))



Genu valgum Normal Genu varum
Figure 1-6 Genu valgum and Genu varum (Adapted from (Roos, Herzog et al. 2011))

1.2.2.1.3 Scoliosis

Scoliosis is a 3D curvature of the spine (Figure 1-7). It can be congenital, neuromuscular, or idiopathic in nature. It affects 2% to 4% of all adolescents whether male or female. However, the chance of curve progression is 10 times higher in female. (Horne, Flannery et al. 2014)

Adolescent idiopathic scoliosis (AIS) is the most common form of scoliosis. The progression of the deformity is governed by the mechanical modulation of vertebral bone growth through the vicious cycle and is more rapid during growth spurt. Treatment options vary based on the degree of severity of the deformity that is measured by the Cobb angle (Figure 1-8). The Cobb angle is the angle between the intersecting perpendicular lines drawn to the lines passing from the top of most tilted vertebra above and the bottom of the most tilted vertebra below the curve.

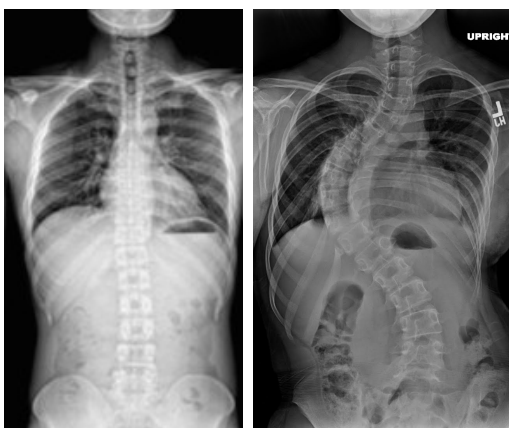


Figure 1-7 Normal spine vs. Scoliotic spine
(Adapted from (AnatomyBox 2013, MEDICS 2015))

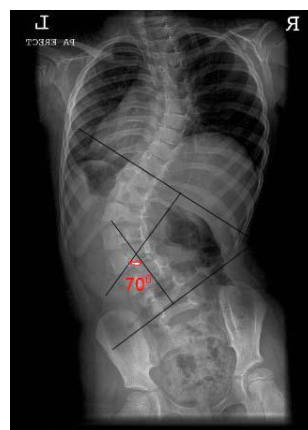


Figure 1-8 Cobb angle (adapted from (Lakshmanan, Ahuja et al. 1015))

For low Cobb angles, only screening and frequent radiography is performed. For moderate Cobb angles, bracing is the treatment of choice (Figure 1-9 A) while in severe cases surgical intervention is performed followed by the fusion of two or several intervertebral levels (Figure 1-9). Fusion of vertebrae in juvenile and adolescents when growth is not complete results in incomplete development of trunk, lungs and heart, which can be lethal. Thus, fusion of vertebrae is not a desirable treatment for early onset or adolescent idiopathic scoliosis (Thompson, Lenke et al. 2007). Recently, the concept of using bone growth potential within the growth plate for correcting the deformities through a fusionless approach has been the center of attentions.

Fusionless treatment approaches used in pediatric orthopedics can be categorized in three groups: 1) methods based on compression of convex side (Braun, Ogilvie et al. 2004, Newton, Faro et al. 2005, Wall, Bylski-Austrow et al. 2005, Guille, D'Andrea et al. 2007, Samdani, Betz et al. 2010, Driscoll, Aubin et al. 2012), 2) methods based on the distraction of the concave side (Guille, D'Andrea et al. 2007, Akbarnia, Mundis Jr et al. 2010, Campbell 2010, Miladi and Dubousset 2010, Thompson and Hing 2010), and 3) growth guiding methods (Luque 1982, McCarthy, Sucato et al. 2010) (Figure 1-9 C).



Figure 1-9 Different treatments of scoliosis. A) Boston Brace (adapted from Grivas et al. 2011). B) Growing Rods (adapted from Akbarnia et al. 2007). C) Vertebal body Stapling (adapted from Akbarnia et al. 2007)

1.2.2.2 Type of mechanical modulation in fusionless treatments

All the fusionless methods developed until now, modulate growth by applying static loading while it was shown in a study by Valteau et al. 2011 that dynamic loading has the same modulating effect as static loading while it causes less damages to the growth plate histomorphology. This implies that fusionless instrumentations applying dynamic loading might be a better solution for treatment of scoliosis and guarantee the normal growth after correction of deformity. Moreover, it was shown in another study that dynamic loading better preserve the protein synthesis of growth plate (Sergerie, Parent et al. 2011). However, the optimal loading parameters that have the most modulating effects while altering the biomechanical and biological characteristics of growth plate tissue to the least extent remain to be clearly characterized.

1.3 Mechanobiology of growth plate

Growth plate chondrocytes, similar to articular cartilage chondrocytes and many other cells responds to their mechanical environment and regulate their synthetic activity according to the forces applied to them (Mow, Gu et al. 2005). As explained in details in section 1.2.2, unbalanced mechanical loading of growth plate can lead to angular deformities. This concept is also used for the development of new fusionless treatments. To better understand the mechanism of growth modulation and in order to find the optimal loading conditions for these fusionless treatments, many studies have investigated the effects of mechanical modulation, especially static vs. dynamic compressive modulation, on growth plate responses. In the following, the effects of different experimented mechanical modulations on growth rate, histomorphology and protein synthesis of growth plate are explained.

1.3.1 Effect of mechanical loading on bone growth rate

To date, many *in vivo* studies have investigated the effect of mechanical modulation of growth plate, explained by Hueter-Volkmann principle, on bone growth rate using different animal models. Growth rate is measured by calculating the growth of bone between two time intervals marked with a fluorochrome such as Calcein that binds to the extracellular matrix during mineralization (Álvarez, Balbín et al. 2000, Farnum, Nixon et al. 2000, Stokes, Gwadera et al. 2005). Based on the results, compressive loading, when under a certain physiological threshold, reduces growth rate (Farnum, Nixon et al. 2000, Stokes 2002, Stokes, Aronsson et al. 2006, Cancel, Grimard et al. 2009) in a magnitude dependent manner (Ohashi, Robling et al. 2002, Stokes, Aronsson et al. 2006). For compressive stresses higher than the threshold, growth stops completely. The value of this threshold was estimated by extrapolation of the average growth reduction per 0.1 MPa increment of compression, where compressive stresses higher than 0.6 MPa stops bone growth (Stokes, Aronsson et al. 2006).

Most of the studies have investigated the effect of static loading on growth rate. In Table 1-3 a summary of *in vivo* studies with static modulation is presented. In general according to all of these studies, static compressive modulation of growth plate reduces longitudinal growth rate of bones. Also, most of the above mentioned studies have investigated the effect of compressive modulation on growth plate morphology in tissue and/or cellular level. According to their results

the overall growth plate thickness is reduced by static compressive modulation [5, 29, 4, 1], which is mostly because of reduced thickness of proliferative zone (less number of proliferative chondrocytes) [29, 4, 1] and hypertrophic zone (reduced height of hypertrophic zone) [29, 4, 1]. In addition it was shown in a study by Cancel et al [5] that type II and X collagen proteins will be reduced under static modulation while MMP-3 mRNA production is increased.

Table 1-3 In vivo studies with static compressive modulation

Paper	Animal Model	Tissue	Static Loading
Cancel, 2009	Rat	Caudal vertebra	0.2 MPa
Stokes, 2007	Rat, Rabbit, calf	Tibia	0.1-0.2 MPa
Stokes, 2006	Rat, Rabbit, calf	Tibia, Caudal vertebra	0.1-0.2 MPa
Stokes, 2005	Rat	Tibia, Caudal vertebra	0.1 MPa
Stokes, 2002	Rat	Caudal vertebra	60% Body mass
Farnum, 2000	Rat	Tibia	Unknown (staple)

Some other in vivo studies have investigated the response of growth plate to dynamic modulation (Table 1-4). According to the results of Niehoff et al., 2004 unlimited or limited exercise reduces growth plate height and number of proliferative chondrocytes, while it does not change collagen type II expression and ultimate shear strength of growth plates. Also, Ohashi et al. 2002 showed that longitudinal growth suppression in response to dynamic loading is magnitude dependent and in higher load magnitudes, mineralization and blood vessel invasion is suppressed. Also, in higher load magnitudes, the morphological parameters such as growth plate height and hypertrophic zone height were significantly greater than control. Moreover, it was shown in the study of Menard et al. 2014 that changing the frequency or amplitude of dynamic loading does not affect the bone growth modulation. However, it was shown in their study that increasing both frequency and amplitude at the same time result in tissue inflammation and the animals had to be sacrificed

Table 1-4 In vivo studies with dynamic compressive modulation

Paper	Animal Model	Tissue	Loading Parameters	Frequency (Hz)
Ohashi, 2002	Rat	Ulnae	4, 8.5, 17 N	2
Niehoff, 2004	Rat	Femor	Unlimited or limited exercise	—
Menard, 2014	Rat	Caudal vertebra	0.2 \pm 30 or 100% MPa	0.1, 1.0

More recently, several other studies have investigated the differential effect of static vs. dynamic loading (Table 1-5). According to the studies which used the same average compression for dynamic and static groups, static and dynamic modulation result in similar growth reductions; however, dynamic loadings is less damaging for the growth plate histomorphology (Valteau, Grimard et al. 2011, Ménard, Grimard et al. 2014). Also, it was shown by (Robling, Duijvelaar et al. 2001) that growth suppression in response to static and dynamic loading is proportional to peak load magnitude and not the average load. Moreover, (Akyuz, Braun et al. 2006) showed that dynamic loading creates a more severe wedge deformity in comparison to static loading. The duration of mechanical modulation also was found to affect growth rate of bones; the longer the load is sustained, the higher will be the growth reduction (Stokes, Gwadera et al. 2005).

Table 1-5 In vivo studies with static and dynamic compressive modulation

Paper	Animal Model	Tissue	Loading parameters	Frequency (Hz)
Valteau, 2011	Rat	Caudal vertebra	0.2±30%	0.1
Akyuz, 2006	Rat	Caudal vertebra	55% B.W.	1
Robling, 2001	Rat	Ulna	8.5-17 N	0.2 Hz

Most of the studies that have investigated growth modulation have used an *in vivo* approach. However, an *in vitro* explant culture, which is extensively used for cartilage studies provide the opportunity to study the effect of modulation in controlled mechanical, and biochemical environment. Moreover, it provides the opportunity for studying the effect of a large number of parameters. In an *in vitro* study by, the effect of high level impact and low level intermittent compression of growth plate on its collagen and proteoglycan metabolic responses has been investigated (Greco, De Palma et al. 1989). It was shown that single high impact compressive force irreversibly decreases proliferation and matrix synthesize while intermittent low-level compressive stresses reversibly enhance proliferation and matrix synthesize. Also, in another study where the response of growth plate to low level compression was investigated, it was shown that low compression reduces synthetic activities of chondrocytes (Mankin and Zaleske 1998).

In a more recent study, the effect of short term static compressive modulation on growth plate biological responses has been studied (Villemure, Chung et al. 2005). According to the results of this study, short-term static modulation of growth plate reduces mRNA expression of type II collagen and type X throughout the growth plate and in hypertrophic zone. To date, the only *in vitro* study that has investigated the differential effect of dynamic and static modulation on growth plate explants is performed by Sergerie et al. 2011. According to the results of this study, static loading reduces aggrecan, type II and type X collagen expression while dynamic loading increase aggrecan and type II collagen protein expression. However, in this study the application of mechanical loading was strain controlled and the level of stress applied to the tissue could not be exactly controlled.

Table 1-6 *In vitro* studies with dynamic and/or static compressive modulation

Paper	Animal Model	Tissue	Loading parameters	Type of loading
Sergerie, 2011	Swine	Ulna	Strain controlled	Static vs. dynamic
Villemure, 2005	Rat	Tibia	55% B.W.	Static
Mankin, 1998	Bovine	Radius	0.012 MPa	Static
Greco, 1989	Rabbit	Femor	High impact/ low intermittent	Static

Although few studies have investigated the effect of mechanical modulation on growth plate responses *in vitro*, numerous studies have been performed on cartilage tissue explants, which is a very similar tissue to growth plate. These studies have investigated the effects of mechanical stimuli, overloading, under-loading, continuous and intermittent static and dynamic modulation on chondrocytes activities and ECM molecule expressions in cartilage. These studies have shown that mechanical load can have both stimulatory and inhibitory affects on biosynthesis of ECM macromolecules and chondrocytes differentiation and proliferation. In general, static, continuous and loads out of physiological range result in reduced matrix production and increased matrix metalloproteinases expression while dynamic, intermittent physiological loads increase synthetic activities.

Some of the notable results from mechanical modulation studies of articular cartilage can be summarized as follow:

Mechanical Modulation Effects on Viability of chondrocytes

After 6 hours of continuous cyclic loading or 3 h of static loading (average stress of 1.0 MPa) cell death occur in superficial tangential zone (Lucchinetti, Adams et al. 2002) and no correlation exist between frequency of intermittence of dynamic load and cell viability while reduction of frequency of intermittence in static loads reduce chondrocyte viability (Wolf, Ackermann et al. 2007). The higher the magnitude of the load, the sooner cell death occurs in superficial tangential zone and in stress levels as low as 0.1 MPa almost no cell death occurs (Chen, Bhargava et al. 2003). Also, viability of dynamically modulated chondrocytes in articular cartilage is independent of the frequency (Chen, Bhargava et al. 2003, Sauerland, Raiss et al. 2003).

Mechanical Modulation Effects on Synthetic Activities of Articular Cartilage

Compressive dynamic and static loading reduce collagen synthesize rate and secretion into medium. There is no relationship between rates of collagen synthesized and frequency of intermittence of load applying, or the load frequency for dynamic loading (Sauerland, Raiss et al. 2003, Ackermann and Steinmeyer 2005, Wolf, Ackermann et al. 2007). Cleaved and denatured collagen will appear in superficial tangential zone after 24 hours of continuous dynamic loading with stress levels of more than 0.1 MPa (Chen, Bhargava et al. 2003). In response to compressive loading, fibronectin content of explants also increase, but its secretion into medium is unaffected by loading (Wolf, Raiss et al. 2003). Compressive dynamic modulation increase proteoglycan synthesize independent from loading magnitude but dependent to unloading period and also dependent to loading duration. In the mean time the total proteoglycan content of loaded explants will remain unchanged (Steinmeyer, Knue et al. 1999). Also, it was observed that in lower frequencies the stimulation of aggrecan synthesis is uniform across the explant while in higher frequencies, mostly peripheral parts are stimulated (Buschmann, Kim et al. 1999).

In response to tensile modulation however, growth rate is accelerated if tension is applied in a physiological range. The increment in growth rate is much lower than the decreasing rate if the same compressive loading is applied (Stokes 2002, Stokes, Aronsson et al. 2006). It is noteworthy to mention that in response to both compressive and tensile modulation, growth

plates from different anatomical locations showed a similar change in growth rate (Stokes, Aronsson et al. 2006).

Very little information is available on the effect of shear and torsion stress on growth rate of bone (Villemure and Stokes 2009). The result of one single study has shown there is no correlation between torsion and bone growth rate (Moreland 1975).

1.3.2 Effect of mechanical loading on cell and tissue histomorphology of growth plate

Growth plate histomorphology is studied both at tissue and cell levels. At cell level, parameters such as hypertrophic cell height and number of proliferative zones are studied. At tissue level, thickness of growth plate and its zones and arrangement of cells are evaluated. Various *in vitro* and *in vivo* studies have evaluated the effects of mechanical modulation on growth plate histomorphology.

It has been shown in previous studies that in response to static compressive modulation *in vivo*:

- the number of proliferative chondrocytes is reduced (Farnum, Nixon et al. 2000, Stokes, Clark et al. 2007, Valteau, Grimard et al. 2011);
- the height of hypertrophic chondrocytes is reduced (Farnum, Nixon et al. 2000, Stokes, Clark et al. 2007, Valteau, Grimard et al. 2011);
- the total growth plate thickness is reduced (Villemure, Chung et al. 2005, Stokes, Clark et al. 2007, Valteau, Grimard et al. 2011);
- the proliferative zone thickness (Stokes, Clark et al. 2007, Valteau, Grimard et al. 2011) and the hypertrophic zone thickness (Farnum, Nixon et al. 2000, Stokes, Mente et al. 2002, Stokes, Clark et al. 2007) are also reduced.

Moreover, the columnar arrangement of chondrocytes is lost both in growth modulation studies performed *in vivo* (Farnum and Wilsman 1998, Valteau, Grimard et al. 2011) and *in vitro* (Sergerie, Parent et al. 2011).

Compressive static loading has been shown to affect the histomorphology of growth plate to a higher extent in comparison to dynamic loading (Valteau, Grimard et al. 2011). In a study comparing static and dynamic compressive modulation with similar magnitude, dynamic

modulation reduced the above mentioned parameters to a lower extent (Valteau, Grimard et al. 2011) compared to static modulation. Moreover, changing the frequency or the amplitude of the dynamic loading does not impact on histomorphological parameters while increasing both parameters at the same time created infection in a study which might be an indicator of a loss of tissue integrity.

In response to tensile static modulation of growth plate:

- growth plate thickness is increased (Alberty, Peltonen et al. 1993, Stokes, Mente et al. 2002);
- columnar arrangement of chondrocytes is lost (Alberty, Peltonen et al. 1993);
- proliferative zone thickness and hypertrophic zone thickness are increased (Alberty, Peltonen et al. 1993);
- the number of hypertrophic chondrocytes and their height are increased (Alberty, Peltonen et al. 1993, Stokes, Mente et al. 2002).

1.3.3 Effect of mechanical loading on ECM proteins and enzyme expressions

A few studies have investigated the effect of mechanical modulation on ECM proteins and degrading enzymes both at the gene and protein levels. It was shown both *in vivo* and *in vitro* that static compressive modulation decreases the expression of type II and type X collagen (Niehoff, Kersting et al. 2004, Villemure, Chung et al. 2005, Cancel, Grimard et al. 2009, Sergerie, Parent et al. 2011). Aggrecan expression, however, remained unchanged in response to static loading *in vivo* while in the *in vitro* study it was reduced (Cancel, Grimard et al. 2009, Sergerie, Parent et al. 2011). Dynamic compressive modulation neither affected collagen expression nor aggrecan expression *in vivo* (Niehoff, Kersting et al. 2004). However, in an *in vitro* study, dynamic loading increased type II collagen expression (Sergerie, Parent et al. 2011).

For enzyme expressions, static loading did not have any effect on MMP-13, ADAMTS-4 and -5 expressions *in vivo* while it was shown to increase MMP-3 (Cancel, Grimard et al. 2009).

1.4 Methods used in mechanobiology of growth plate and cartilaginous tissues

1.4.1 Methods used for mechanical characterization

Most of the studies on growth plate mechanobiology investigate its mechanical properties in compression. In order to be able to study the time dependent behaviour of growth plate under compression, the stress relaxation or creep can be tested in confined compression, unconfined compression or indentation.

In confined compression, the cylindrical sample is closely surrounded by an impermeable confining chamber and the compression is applied using a porous platen (Figure 1-10 A). As a result, fluid exudation only occurs from the platen in the axial direction. However, it is hard to achieve the confined compression experimentally as it is hard to prepare the sample exactly to the size of confining chamber.

In unconfined compression, the tissue is in contact with impermeable platens on its upper and lower surfaces, while it is free to swell from circumferential sides and the fluid can exude in radial direction (Figure 1-10 B). *In vivo*, the growth plate is located *between* epiphyseal and metaphyseal bones from its upper and lower sides and is in contact with tendons and soft tissue from circumferential sides. Fluid exudation can then happen in all the directions. Therefore, by using two porous filters or by keeping the epiphyseal and metaphyseal bones on the sample, fluid exudation can occur in all the directions as well.

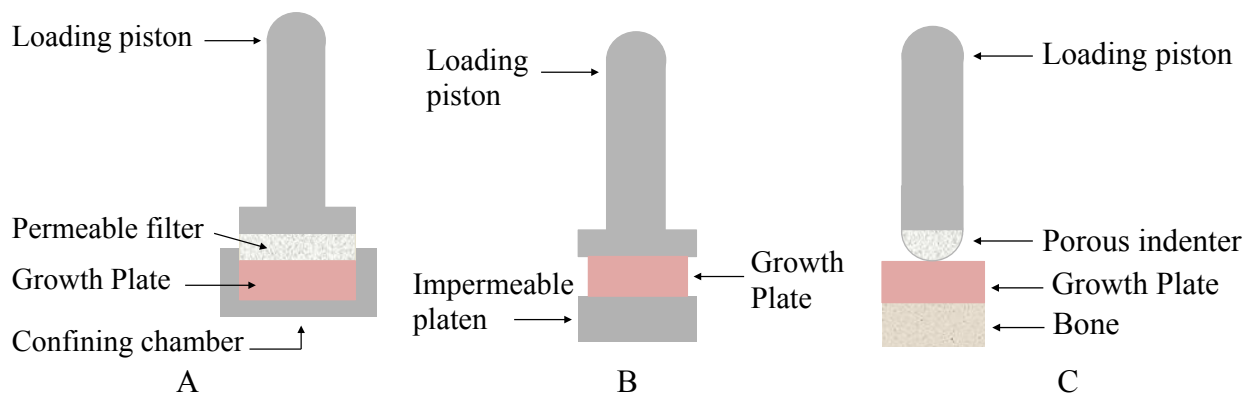


Figure 1-10 Cartilaginous tissue mechanical compressive test setup

In indentation, a compressive force is applied locally to the tissue using either a permeable or impermeable indenter (Figure 1-10 C). This method does not need a specific protocol for preparation of samples, but it is difficult to standardize the models for extraction of mechanical parameters using experimental data, where a calibration procedure is required.

Stress-relaxation responses from confined and unconfined compressive tests have been previously fitted to non-linear biphasic (Cohen, Chorney et al. 1994), transversely isotropic biphasic (Cohen, Lai et al. 1998, Sergerie, Lacoursiere et al. 2009, Wosu, Sergerie et al. 2012) or fibril-network reinforced biphasic (Ménard, Soullisse et al. 2014) models.

In order to study the zone dependent response of growth plate in compression, the stress relaxation test can be combined with confocal microscopy and digital image correlation to find strain patterns of tissue under compression (Villemure, Cloutier et al. 2007, Amini, Mortazavi et al. 2013).

1.4.2 Biological characterization of growth plate and cartilaginous tissues

1.4.2.1 Histomorphometry

Many studies have addressed the two dimensional histomorphology of growth plate in normal state and after mechanical modulation. Different histomorphological parameters such as the thickness of the growth plate and its zones, the number of proliferative and hypertrophic cells and the height of hypertrophic cells are calculated using semi-automatic measurements of the parameters on histological images (Cancel, Grimard et al. 2009, Sergerie, Parent et al. 2011, Valteau, Grimard et al. 2011, Ménard, Grimard et al. 2014).

An alternative method that has been recently used in the literature is the 3D reconstruction of serial 2D confocal images (Guilak 1994, Amini, Veilleux et al. 2010). Using this method, the changes in volume and surface of the chondrocytes can be evaluated.

1.4.2.2 Viability

Viability of chondrocytes in growth plate has not been studied extensively. However, the viability of chondrocytes in articular cartilage, which is very similar to growth plate, has been extensively studied. Most studies have used live/dead viability staining, which consists of two fluorophores; a widely used example of this method is using Calcein Am and Ethidium

Homodimer 1. Calcein Am stains the live cells based on the esterase activity in cytoplasm and produce a green light, Ethidium Homodimer 1 on the other hand only enters the cells with compromised membrane, binds to the nuclei and produces a red fluorescence. Other methods based on cellular metabolic activity such as MTT have also been used in the literature.

1.4.2.3 Protein

Proteins in cartilaginous tissue can be detected and/or quantified using western blot, immunohistochemistry and biochemical content evaluation methods.

The *Western Blot* technique is a semi-quantitative method, which can detect specific proteins in a tissue based on their size. In this method, proteins are separated using gel electrophoresis based on their polypeptides length or based on their three-dimensional structure and are transferred to a membrane. Using the desired antibodies, proteins can be detected and later revealed radiography, fluorescence or radioactive detection (Kurien and Scofield 2006). By comparing the amount of protein detection with a known standard, the quantity of protein can be estimated. This method does not give any information on the location of proteins. This technique has not been used much in growth plate studies.

Immunohistochemistry (IHC) is a method that can detect specific proteins in a tissue. In this method using a chain of chemical reactions, specific antibodies bind to the specific proteins. The detection and visualization can be done either by chromogens or immunofluorescence (Nerlich 2003). This method gives information on localization of the protein but it is not quantitative. Recently, many studies have used digital image processing methods to quantitate the reaction signals. This method has been used extensively in the literature to detect proteins in growth plate.

Biochemical content evaluation

Biochemical content evaluation is a general name for a series of colorimetric methods. These assays are based on changes in the absorption spectrum of a dye when bound to a special component of the target protein. The changes in the absorbance are measured using plate reader photometers and are compared with a standard curve to estimate the quantity of samples. The widely used biochemical assays for growth plate and articular cartilage are DMMB assay and Hydroxyproline assay which respectively detect GAG and collagen. This method is quantitative

but again the localization of proteins is not possible. Moreover, it is not specific and it detects all the proteins in a family. (Barbosa, Garcia et al. 2003, Hoemann 2004)

CHAPTER 2 PROJECT RATIONALE, HYPOTHESES AND OBJECTIVES

2.1 Rationale

Based on the review of the literature, the rationale of the project can be explained as follow:

- The growth plate is a cartilaginous tissue consisting of chondrocytes embedded in an extracellular matrix rich in collagen and proteoglycan.
- The growth of long bones and vertebrae is achieved by a synchronized differentiation and proliferation of chondrocytes and matrix synthesis.
- The mechanical modulation of bone growth is the underlying factor of progression of angular deformities based on Hueter-Volkman principle.
- The treatment of progressive angular deformities can be performed using the mechanical modulation of bone growth.
- Efficient and non-damaging mechanical loading parameters for the mechanical modulation of bone growth are not clearly defined.
- Static and dynamic compressions have a similar effect on bone growth rate but different effects on histomorphometry and protein expression of growth plate, where dynamic loading seems to be less detrimental.
- Mechanical responses of growth plate in compression are zone based and mostly dependent on its collagen and proteoglycan contents.
- The effects of mechanical modulation on the growth plate biomechanical properties remain to be investigated.

2.2 Hypotheses

H1. Static and dynamic modulations trigger different effects on chondrocyte viability, and static modulation is more detrimental for cellular viability.

H2. Statically and dynamically modulated growth plate explants show different changes in their biomechanical properties and dynamically modulated growth plate explants remain more similar to baseline samples.

H3. Among dynamic modulating parameters, frequency causes more significant changes in growth plate biomechanical properties, viability and extracellular matrix integrity compared to loading magnitude.

H4. Changes in growth plate biomechanical properties and chondrocyte viability are associated with changes in extracellular matrix integrity and tissue and cell histomorphometry.

2.3 Objectives

The overall objective of this project was to evaluate the effects of different mechanical modulation parameters on the biomechanical and biological responses of growth plate tissues. In the following sections, the four specific objectives, which helped verifying the hypotheses, are presented.

Objective 1: To investigate the effect of different static/dynamic compressive modulation parameters on the viability of growth plate chondrocytes. This objective had four main steps:

1. To identify the optimal concentration for live/dead florescent labeling of viable and dead chondrocytes and the optimal incubation time for swine ulnar growth plate explants.
2. To optimize the composition of a culture medium to maximize chondrocytes viability.
3. To implement ten groups of growth plate explants (a) baseline, (b) culture control, (c) three static modulation groups (two different magnitudes and durations), (d) five dynamic modulation groups (two different magnitude, duration, frequency, amplitude) using the parameters listed in Table 2-1.
4. To visualize and quantify for each group, the number of live and dead cells using an automatic cell quantification method.

The first step involved a preliminary study of different concentrations of live/dead fluorescent probes and their incubation time in order to determine the optimal concentration and duration.

The second step involved finding the right components and their concentration for growth

plate explant culturing. Different concentrations of bovine serum and antibiotics (penicillin and streptomycin) combined to DMEM were tested. In the third step, ten different groups of experiment were prepared. Baseline samples were fresh samples and were tested for viability right after dissection. Culture control samples were incubated in culture medium without mechanical modulation. Three static groups and five dynamic groups were prepared by mechanical modulation of samples using the parameters listed in Table 2-1. In the last step, all the explants were labeled with live/dead viability assay fluorescent probes and confocal images were taken. Number of live and dead cells were quantified using a gradient flow tracking quantification algorithm, which was developed for quantification of number of cells in fluorescent images with closely packed cells (Kaviani, Merat et al. 2015).

Table 2-1 Mechanical modulation parameters for viability test

Groups		Stress	Amplitude	Frequency	Loading Duration
Baseline Control Static			Fresh samples Cultured for 48 hours		
	Stat1	0.1	–	–	12
	Stat2	0.2	–	–	12
Dynamic	Stat3	0.1	–	–	24
	Dyn1	0.1	30%	0.1	12
	Dyn2	0.2	30%	0.1	12
	Dyn3	0.1	30%	0.1	24
	Dyn4	0.1	30%	1.0	12
	Dyn5	0.1	100%	0.1	12

Objective 2: To evaluate, the effect of different static/dynamic compressive modulation on the biomechanical properties in terms of tissue strain patterns, equilibrium and maximum stresses, matrix modulus, fibril modulus and permeability.

This objective consisted of three steps:

- 1) To implement 6 groups of growth plate explants (a) baseline, (b) culture control, (c) static (d) three dynamic modulation groups (using the parameters listed in Table 2-2).

- 2) To label each semi-cylindrical explant with Syto 17 nucleic acid fluorophore, perform a stress relaxation test and take confocal images before loading of the explant and after relaxation using a custom developed micromechanical testing machine installed on the stage of an inverted confocal microscope.
- 3) To calculate the strain field within each sample using a digital image correlation algorithm applied on the two images taken in the previous step.
- 4) To fit the data of stress relaxation test with a fibril-network reinforced biphasic model to find the matrix modulus, fibril modulus and permeability of the explant.

At first, based on the results of the previous objective, experimental groups, which showed acceptable viability, were chosen for the continuation of the study. Baseline, culture control, static groups and three dynamic groups were modulated using the parameters listed in Table 2-2. Next step involved preparation of samples for confocal imaging and performing the stress relaxation test. The stress relaxation test was performed using a custom developed mechanical testing setup installed on the stage of an inverted confocal microscope so that at the same time the loading could be applied and the images could be taken. Digital 2-D images of the labeled nuclei were taken prior to stress relaxation and after tissue relaxation. In the third step, a digital image correlation algorithm was used to calculate the strain pattern through the thickness of the growth plate. Strain developed within each zone was further calculated by averaging the data within the three zones. In the last step, data of the stress relaxation test were fitted on a fibril-network reinforced model and the matrix modulus, fibril modulus and permeability were obtained. Equilibrium and maximum stresses were extracted from the force sensor data.

Objective 3: To characterize, for the explants of each group, tissue histomorphometry in terms of the thickness of each zone and the thickness of growth plate.

In this objective, histomorphometry was quantified using a semi-manual image processing method on toluidine blue stained MMA sections.

Objective 4: To characterize the extracellular matrix integrity in terms of proteoglycan and collagen content and expression of aggrecan, type II and X collagens.

This objective had four steps.

- 1) To divide each frozen part of the explants into its three constituting zones (reserve, proliferative and hypertrophic) based on the histomorphological information acquired in objective 3.

Table 2-2 Mechanical modulation parameters for mechanical characterization, histomorphometry and protein evaluation

Groups	Stress	Amplitude	Frequency	Loading Duration
Baseline				
Control				
Static				
	Stat	0.1	—	12
Dynamic				
	Dyn1	0.1	30%	12
	Dyn2	0.1	30%	12
	Dyn3	0.1	100%	12

- 2) To quantify the GAG content and collagen content of each zone of each sample using biochemical content assays.
- 3) To quantify protein expression for aggrecan types II and X collagen using semi quantitative immunohistochemistry.
- 4) To conduct a comparative study to characterize the differential effects of static vs. dynamic modulation and dynamic loading parameters on chondrocyte viability, biomechanical responses, ECM integrity and histomorphometrical parameters.

In the first step, each frozen part of each explant was divided into its three zones (reserve, proliferative and hypertrophic) using cryosectioning. In the second step, each zone of each sample was digested in papain. For GAG content evaluation DMMB colorimetric assay was used. For collagen content evaluation, the digested samples were further hydrolyzed in HCl and Hydroxyproline colorimetric assay was used. In the third step, using immunohistochemistry, three antibodies of aggrecan, type II collagen and type X collagen, the expression of these proteins and the location of the expression were identified on paraffin tissue sections. The reaction signal intensity of the immunohistochemistry images was quantified using a custom developed algorithm. Finally, the relationship between the effects of mechanical modulation on

changes in the mechanical behaviour of the growth plate, viability, histomorphology, biocomposition and protein expression was evaluated using a comparative study.

The three articles, which forms the body of this thesis, address the four objectives as shown in the following diagram:

Article 1 (Chapter 3)		Article 2 (Chapter 4)		Article 3 (Chapter 5)	
Objective 1		Objective 2	Objective 3	Objective 4	
Hypothesis 1	Hypothesis 3	Hypothesis 2	Hypothesis 3	Hypothesis 3	Hypothesis 4

CHAPTER 3 ARTICLE #1 COMPRESSIVE MECHANICAL MODULATION ALTERS THE VIABILITY OF GROWTH PLATE CHONDROCYTES IN VITRO

This chapter introduces the first article written in the context of this thesis and responds to the first objective of this thesis as detailed in Chapter 2.

The revised manuscript of this article was submitted to *Journal of Orthopaedic Research* © on 27th March 2015 and was subsequently accepted on 25th of May 2015.

The contribution of the first author in the preparation, obtaining the results, writing and literature review of this paper is estimated at 85%.

Contribution of all authors:

Rosa Kaviani: Design of experiment, obtaining the results, analysis, interpretation of results, article writing and editing, responsible for the integrity of the work.

Irene Londono: Technical help (developing protocol for live/dead staining and imaging)

Stefan Parent: Design of experiment, Review of the article.

Florina Moldovan: Design of experiment, interpretation of results, review of the article, responsible for the integrity of the work.

Isabelle Villemure: Design of experiment, interpretation of results, review of the article, responsible for the integrity of the work.

3.1 Abstract

The aim of this study was to investigate the effect of compressive modulation parameters (mode, magnitude, duration as well as frequency and amplitude for cyclic modulation) on the viability of growth plate chondrocytes. Swine ulnar growth plate explants (n=60) were randomly distributed among 10 groups: baseline (n=6); culture control (n=6); static (n=3×6) and dynamic (n=5×6). Static and dynamic samples were modulated *in vitro* using a bioreactor. Different compression magnitudes (0.1 MPa or 0.2 MPa), durations (12 hours or 48 hours), frequencies (1.0 Hz or 2.0 Hz) and amplitudes (30% or 100%) were investigated. Viability was assessed by automatic quantification of number of live/dead cells from confocal images of Live/Dead labeled tissues. Chondrocyte viability was found to be dependent on compression magnitude, duration, frequency and amplitude in a way that increasing each parameter decreased viability in certain zones of growth plate. More specifically, proliferative and hypertrophic chondrocytes were found to be more sensitive to the applied compression. This study provides an *in vitro* protocol for studying the effects of compressive modulation on biomechanical and biological responses of growth plate explants, which will be useful in finding efficient and non-detrimental parameters for mechanical modulation of bone growth exploited in scoliosis fusionless treatments.

3.2 Keywords

Growth plate, chondrocytes, viability, mechanical modulation, static, dynamic

3.3 Introduction

Based on many clinical and experimental evidences (Stokes, Spence et al. 1996, CANCEL, Grimard et al. 2009, Valteau, Grimard et al. 2011), mechanical loading is known to be one of the factors regulating longitudinal bone growth. Longitudinal growth of long bones and vertebrae occurs by continuous calcification of newly produced cartilaginous tissue in the growth plate (R Tracy and Regis 2003). Growth plate is a cartilaginous tissue located at both ends of long bones and vertebrae. It is mainly composed of an extracellular matrix containing one cell type, chondrocytes, which are in three different stages of differentiation forming three distinct zones (Ballock and O'Keefe 2003): the reserve (R), proliferative (P) and hypertrophic (H) zones (R Tracy and Regis 2003). The bio-composition (Amini, Mortazavi et al. 2013) and mechanical

properties (Villemure, Cloutier et al. 2007, Sergerie, Lacoursiere et al. 2009, Amini, Mortazavi et al. 2013) of these three zones are different and associated with specific roles in the growth process.

According to Hueter-Volkmann's principle, bone growth rate is reduced in response to increased compression on growth plate and it is increased in response to decreased compression. This phenomenon, known as the mechanical modulation of bone growth, is considered one of the main causes of progression of developmental growth angular deformities such as scoliosis, genu varum and valgum, following asymmetrical loading of growth plates after the onset of the deformities (Stokes, Spence et al. 1996)

The mechanical modulation of bone growth is also exploited in the development of new fusionless methods for the treatment of adolescent idiopathic scoliosis or genu valgum by reversing the loads distribution on growth plates (Stevens, Maguire et al. 1999, Guille, D'Andrea et al. 2007). The underlying concept of these treatments is retarding growth by increasing the compression on the convex side of an angular deformity, which has a faster growth rate, and conversely, releasing compression on the concave side growing at a slower rate.

Few *in vivo* and *in vitro* studies have previously investigated the effects of compressive static and dynamic modulation on bone growth rate (Cancel, Grimard et al. 2009, Valteau, Grimard et al. 2011, Ménard, Grimard et al. 2014), histomorphology (Valteau, Grimard et al. 2011, Ménard, Grimard et al. 2014) and protein expression (Cancel, Grimard et al. 2009, Sergerie, Parent et al. 2011). During mechanical modulation, growth plate chondrocytes undergo mechanical stimulation; they need to survive this mechanical stimulus in order to maintain their matrix integrity, which in turn will determine the resulting mechanical properties of the tissue. Based on previous studies on articular cartilage, which is a very similar to growth plate in terms of structure and biocomposition, chondrocytes viability in explants modulated *in vitro* was found dependent on the mode of loading (static or dynamic) (Lucchinetti, Adams et al. 2002), magnitude (Chen, Bhargava et al. 2003, Levin, Chen et al. 2005), duration (Chen, Bhargava et al. 2003, Levin, Chen et al. 2005) and zone (Chen, Bhargava et al. 2003, Levin, Chen et al. 2005) but non dependent on frequency (Sauerland, Raiss et al. 2003). However, to date no study has investigated the effects of mechanical modulation on chondrocyte viability in growth plate explants. The goal of this study was to systematically investigate the effects of the mode of

compressive modulation (static or dynamic) as well as its magnitude and duration and, for dynamic loading, the effect of its frequency and amplitude, on the viability of *in vitro* mechanically modulated growth plate explant chondrocytes. This study was conducted to further design an experimental protocol for extensive studies on the effects of mechanical modulation on the biomechanical and compositional responses of growth plates.

3.4 Methods

3.4.1 Sample Preparation

Forelegs from 4-week-old swines were obtained from a local abattoir within 2 h post mortem. Epiphysis-Growth plate-Metaphysis explants were harvested aseptically from the distal ulnae using a 6-mm diameter biopsy punch (Figure 3-1 A). The upper and lower surfaces of the explants were trimmed using a Vibratome (Vibratome 1500 Sectioning System) to obtain 9 mm thick explants with two parallel surfaces. The thickness of growth plate part was evaluated to be $2581.40 \pm 461.64 \mu\text{m}$ using histomorphological measurements.

Table 3-1 Experimental groups and mechanical modulation parameters

Groups	Stress (MPa)	Amplitude	Frequency (Hz)	Loading Duration (hours)
Baseline	Fresh samples			
Control	Cultured for 48 hours			
Static				
Stat1	0.1	–	–	12
Stat2	0.2	–	–	12
Stat3	0.1	–	–	24
Dynamic				
Dyn1	0.1	30%	0.1	12
Dyn2	0.2	30%	0.1	12
Dyn3	0.1	30%	0.1	24
Dyn4	0.1	30%	1.0	12
Dyn5	0.1	100%	0.1	12

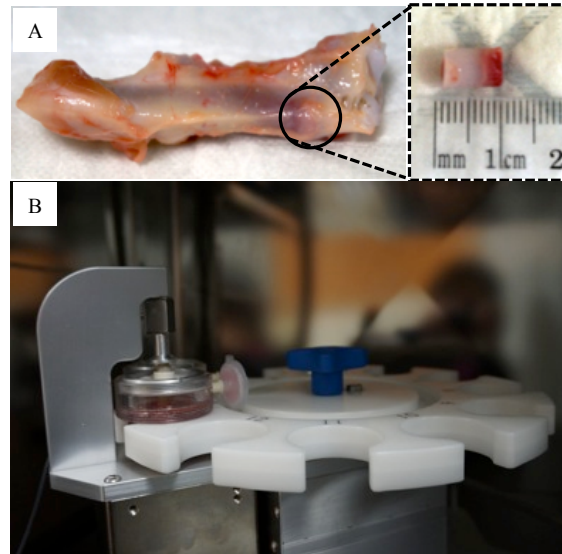


Figure 3-1 Sample preparation and mechanical modulation. A) Growth plate cylindrical explant
B Bioreactor

3.4.2 Mechanical Loading Protocol

The parameters used for the mechanical modulation of explants are summarized in Table 3-1. A preload of 0.001 MPa (0.028 N) representing the weight of the loading piston was applied to all the cultured explants. All cultured explants (culture control or mechanically modulated samples) were cultured for 48 hours. Before applying any mechanical modulation for the loaded groups, the samples were cultured 30 min with the preload to reach a state of equilibrium. Static and dynamic explants were loaded in the direction of growth in radially unconfined compression using the bioreactor linear motor (resolution 0.01 mm) and the degree of compression was controlled using the integrated load cell (resolution 0.01 N) in the system (Figure 3-2). The epiphyseal and metaphyseal parts of the explants were maintained attached on the explant to fulfill the role of physiological porous filter.

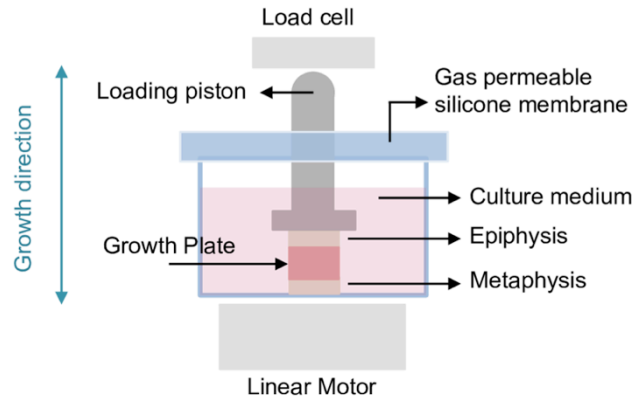


Figure 3-2 Bioreactor chamber with sample modulated in the direction of growth

Static loading was applied with an average compressive stress of 0.1 or 0.2 MPa (Fig. 3). Similar to previous *in vivo* studies, the 0.2 stress level was chosen so that it would be in a biological range that modulates the growth rate without stopping it (Valteau, Grimard et al. 2011, Ménard, Grimard et al. 2014). Dynamic loading was applied using a sinusoidal waveform with an average compressive stress of 0.1 or 0.2 MPa and with a frequency of 0.1 or 1.0 Hz and an amplitude (i.e. variation around the average stress) of $\pm 30\%$ or $\pm 100\%$. The mechanical modulation was performed for a total duration of either 12 or 24 hours over a 48 hours period of culture with parameters chosen based on the group of experiment (Figure 3-3).

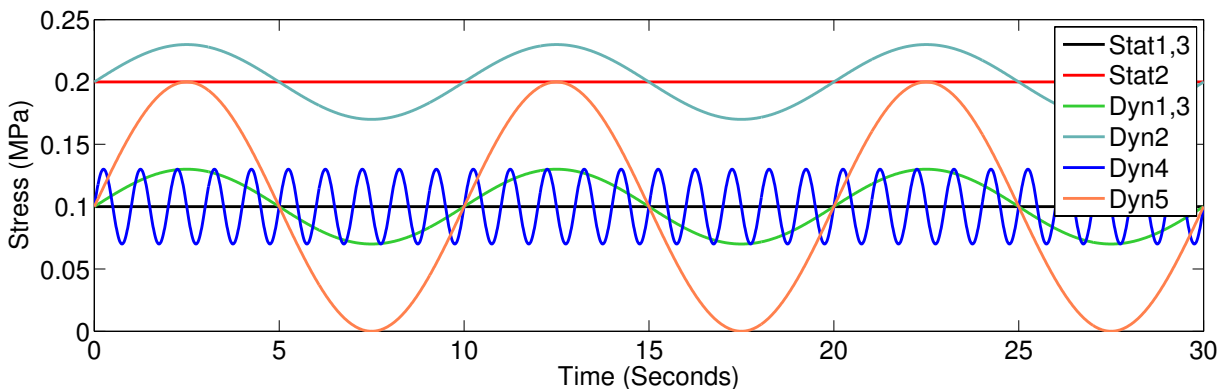


Figure 3-3 Modulation parameters for the different experimental groups

For all the samples modulated for 12 hours (Stat1, Stat2, Dyn1, Dyn2, Dyn4, Dyn5), the loading was applied for 12 hours out of 48 hours of culture with the pattern depicted in Figure 3-4 A (i.e. for each 8 hours, 2 hours loading was completed and then loading was removed for 6 hours). A similar pattern was followed for the samples modulated for 24 hours during 48 hours of culturing as depicted in Figure 3-4 B (i.e. for each 4 hours, 2 hours loading was completed and then

loading was removed for 2 hours). During unloading periods, the load was removed and only the preload of 0.001 MPa was maintained. Control explants were cultured in the same chambers as loaded explants for 48 hours, with only the preload applied.

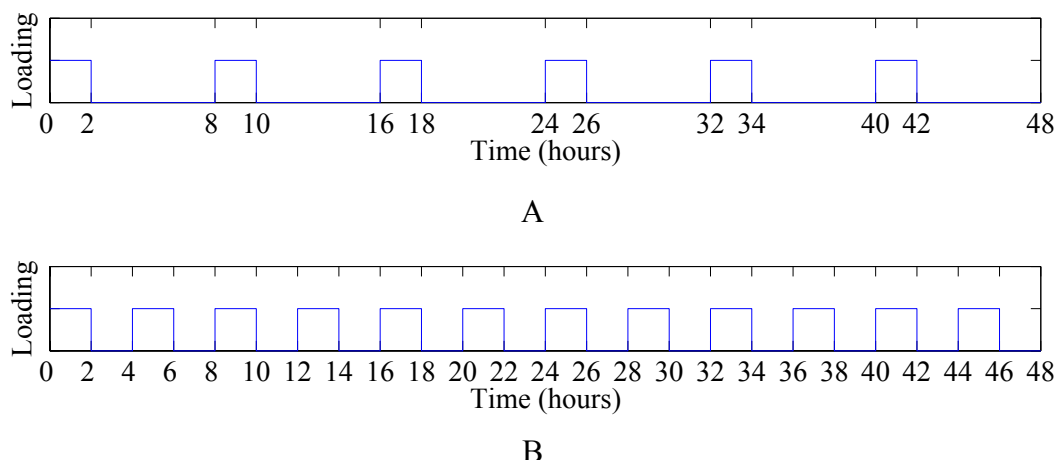


Figure 3-4 Loading and unloading sequence for 12 hour and 24 hour modulated samples. A) 12 hour mechanical modulation periods. B) 24 hour mechanical modulation periods (The square wave only shows the presence or absence of loading regardless of type of modulation)

3.4.3 Viability Assessment

Following the 48 hours incubation period, or right after dissection for baseline samples, a 1 mm thick section was cut from the central part of each explant for viability assays. The rest of the explants were conserved with the appropriate method for future studies on the mechanical and biological characterizations.

3.4.4 Staining

Cell viability was determined using the LIVE/DEAD[®] viability kit (Molecular Probes, Invitrogen, Montreal, Canada). The samples were stained using a solution of 1 μ M Calcein Am and 2 μ M Ethidium Homodimer 1 for 30 minutes at 37 °C. Sections were washed in Hank's balanced salt solution (HBSS, Life Technologies) before imaging.

3.4.5 Imaging

A 100 μm thick image spanning the three zones of the growth plate was taken from serial sections of 1024×1024 pixels using stacks of z-series (serial optical sections of 10 μm). An inverted laser scanning confocal microscope (LSM 510, Carl Zeiss, Jena, Germany) was used for imaging. Images were collected using a 10X 0.30NA Plan-Neofluar lens (Carl Zeiss Inc., Germany). The 488 nm line of an Argon laser was used for Calcein AM excitation combined with a band-pass 500-550 while the 543 nm line of a He-Ne laser was used for Ethidium Homodimer 1 excitation together with a low-pass 560 filter. Maximum projection was used to obtain a planar 2D image of the cells. Following acquisition, a 800×400 region of interest (ROI) was selected from each image for quantification of live and dead cells. All images were filtered with a Gaussian 2D filter for noise smoothing prior to further image analysis.

3.4.6 Quantitative Analysis

The number of live and dead cells were quantified in each image using an improved gradient flow tracking method (Kaviani, Merat et al.). In fluorescent images, cells are convex objects and all their surrounding gradient vectors are pointing toward their centers. Our cell counting method used the gradient vector field of the image to detect the center of convex shapes. The algorithm started with a set of arbitrary initial points on the image and at each iteration, each point was moved in the direction of the average gradient of its vicinity area, which was chosen considering the shape of cells. Finally, the algorithm was stopped when the points reached an equilibrium state (the convergence centers of the gradient vectors). Using appropriate merging and thresholding filtering, closely located points and low intensity points were removed. The remaining points could be associated with cell centers. For each ROI, the number of cells was quantified in four different zones of the growth plate: the hypertrophic zone (H), the proliferative zone (P), the late reserve zone (R.L.) and the early reserve (R.E.) zone (Figure 3-5). The viability of each region was calculated by dividing the number of live cells to the total number of cells (i.e. live plus dead cells) in that ROI.

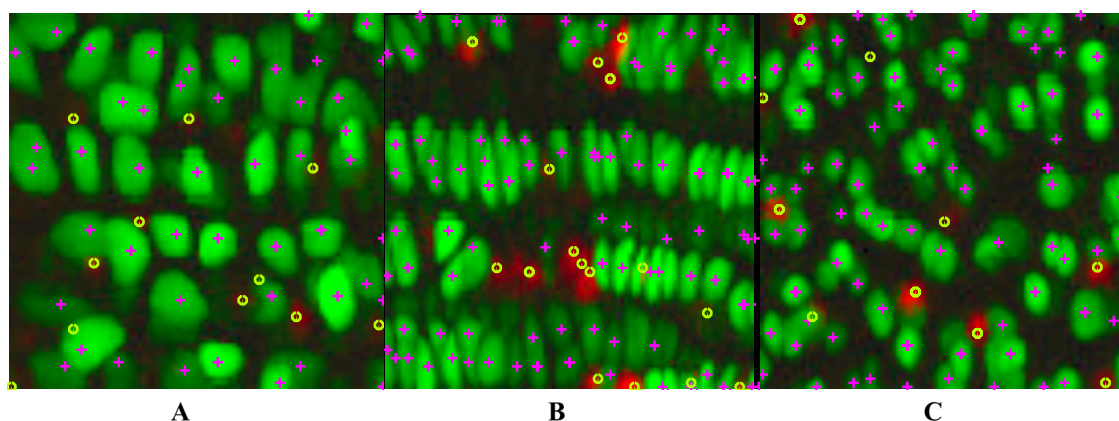


Figure 3-5 Live (green) and dead (red) cells detected by the gradient flow tracking method. Detected live cells are marked by + and detected dead cells are marked by o. A) Hypertrophic zone. B) Proliferative zone. C) Reserve zone

3.4.7 Statistics

All results are reported as means \pm standard deviations of viability percentages in each of the aforementioned zones. One-way analysis of variance (ANOVA) and post hoc comparison using Tukey's method were used to determine the significant differences between groups. *P* values less than 0.05 were considered as significant. Data were analyzed using the MATLAB statistical toolbox (2014a, The MathWorks, Inc., Natick, Massachusetts, United States).

3.5 Results

Results of viability assays using Calcein Am and Ethidium Homodimer 1 staining are shown in (Figure 3-6). The positive staining with Ethidium Homodimer (red staining) indicates dead cells and positive staining with Calcein Am (green staining) indicates live cells.

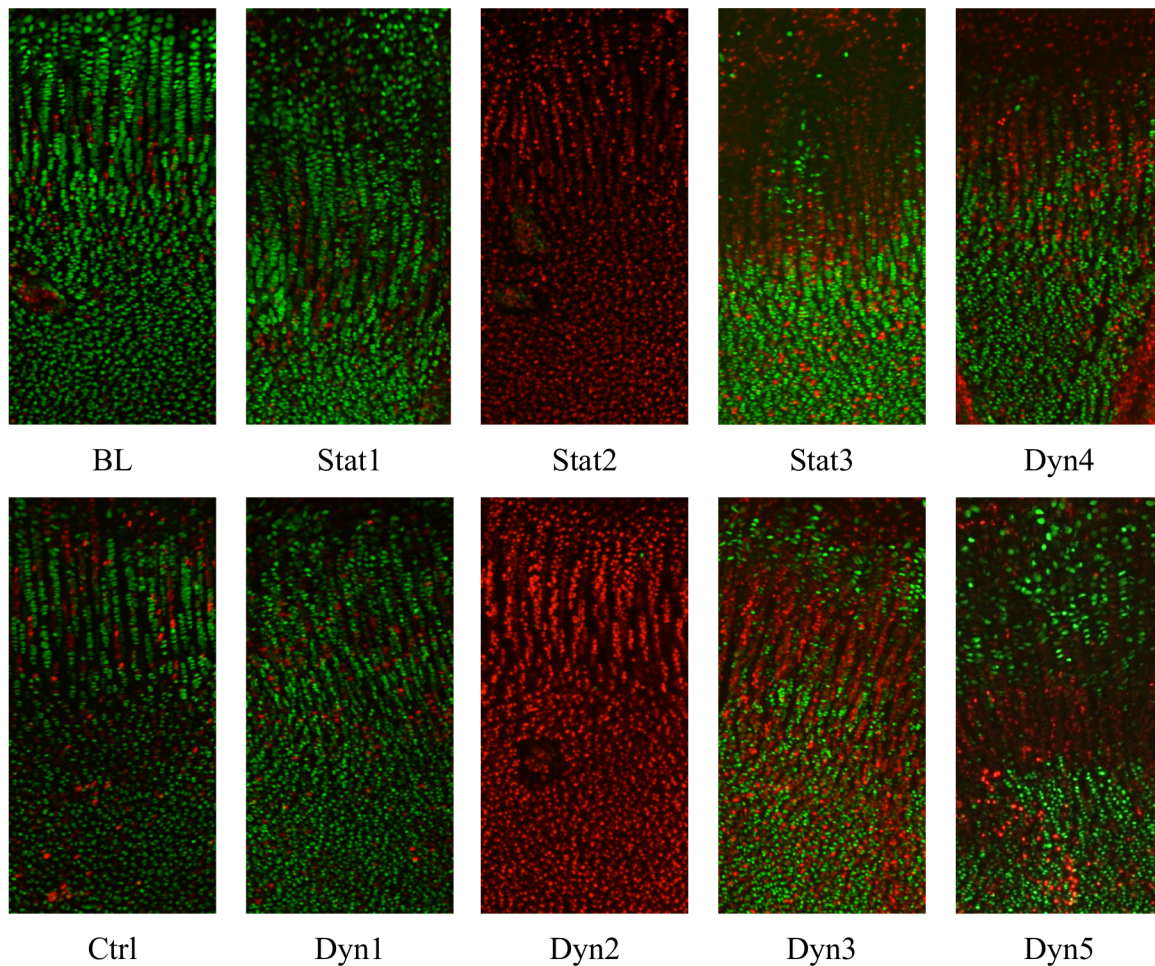


Figure 3-6 Viability of chondrocytes for different groups. Green labeling indicates live cells while red labeling indicates dead cells. BL: Baseline, Ctrl (control), Stat1 (0.1 MPa, 12 hours), Stat2 (0.2 MPa, 12 hours), Stat3 (0.1 MPa, 24 hours), Dyn1 (0.1 MPa \pm 30%, 0.1 Hz, 12 hours), Dyn2 (0.2 MPa \pm 30%, 0.1 Hz, 12 hours), Dyn3 (0.1 MPa \pm 30%, 0.1 Hz, 24 hours), Dyn4 (0.1 MPa \pm 30%, 1.0 Hz, 12 hours), Dyn5 (0.1 MPa \pm 100%, 0.1 Hz, 12 hours)

Average viability percentage for each of the four mentioned zones of growth plate are summarized in Table 3-2 for all experimental groups. In order to isolate the culture effect, control explants were compared with baselines. Chondrocytes viability was significantly reduced by 30%, 13% and 14%, respectively in the hypertrophic, proliferative and late reserve zones of control explants compared to baselines (Figure 3-7 A-C). Viability in the early reserve zone of control explants remained unchanged compared to the baselines (Figure 3-7 D).

Table 3-2 Viability percentages for the different zones of growth plate explants (average \pm SD)

Group	Treatment	Zone of growth plate			
		H (%)	P (%)	R.L. (%)	R.E. (%)
Baseline	Fresh samples	90 \pm 3	80 \pm 5	85 \pm 6	88 \pm 4
Ctrl	Cultured samples	63 \pm 4	70 \pm 3	73 \pm 7	84 \pm 5
Stat1	0.1 MPa, 24 h	63 \pm 11	65 \pm 12	72 \pm 11	86 \pm 6
Dyn1	0.1 \pm 30% MPa, 24h, 0.1 Hz	61 \pm 7	60 \pm 11	68 \pm 9	84 \pm 6
Stat2	0.2 MPa, 24 h	0	0	0	0
Dyn2	0.2 \pm 30% MPa, 24h, 0.1 Hz	0	0	0	0
Stat3	0.1 MPa, 48 h	2 \pm 3	5 \pm 9	0	80 \pm 7
Dyn3	0.1 \pm 30% MPa, 48h, 0.1 Hz	43 \pm 13	33 \pm 9	64 \pm 4	81 \pm 6
Dyn4	0.1 \pm 30% MPa, 24h, 1.0 Hz	33 \pm 3	54 \pm 7	66 \pm 8	84 \pm 7
Dyn5	0.1 \pm 100% MPa, 48h, 0.1 Hz	56 \pm 6	35 \pm 13	71 \pm 8	87 \pm 4

In order to isolate the compressive loading effect, the mechanically modulated explants (static and dynamic) were compared to control explants. When using an average stress of 0.1 MPa for 12h/48h, no significant differences were observed in chondrocytes viability for the four investigated zones between controls and static (Stat1) or dynamic with 0.1 HZ frequency and 30% amplitude (Dyn1) explants (Figure 3-7 A-D). However, when the average stress was increased to 0.2 MPa for the same duration of 12h/48h, viability of all four zones was reduced to zero for both static (Stat2) and dynamic explants with 0.1 HZ frequency and 30% of amplitude (Dyn2) (Figure 3-7 A-D).

When increasing the duration of mechanical modulation to 24h/48h with an average stress of 0.1 MPa, viability of the chondrocytes of static group (Stat3) was reduced by 96%, 92% and 100% in the hypertrophic, proliferative and late reserve zones, respectively with respect to Stat1, while the viability of early reserve zone remained unchanged (Figure 3-7 A-D). However, the dynamic modulation for 24 h/48h with 0.1 MPa of average stress (Dyn3) reduced the viability of hypertrophic and proliferative zones by 29% and 45% respectively, while no significant change was observed in the viability of late and early reserve zones with respect to Dyn1 group (Figure 3-7 A-D).

Increasing the frequency of dynamic modulation from 0.1 Hz to 1.0 Hz with 0.1 MPa \pm 30% stress applied for 12h/48 h (Dyn4) reduced the viability of hypertrophic chondrocytes to 54% of

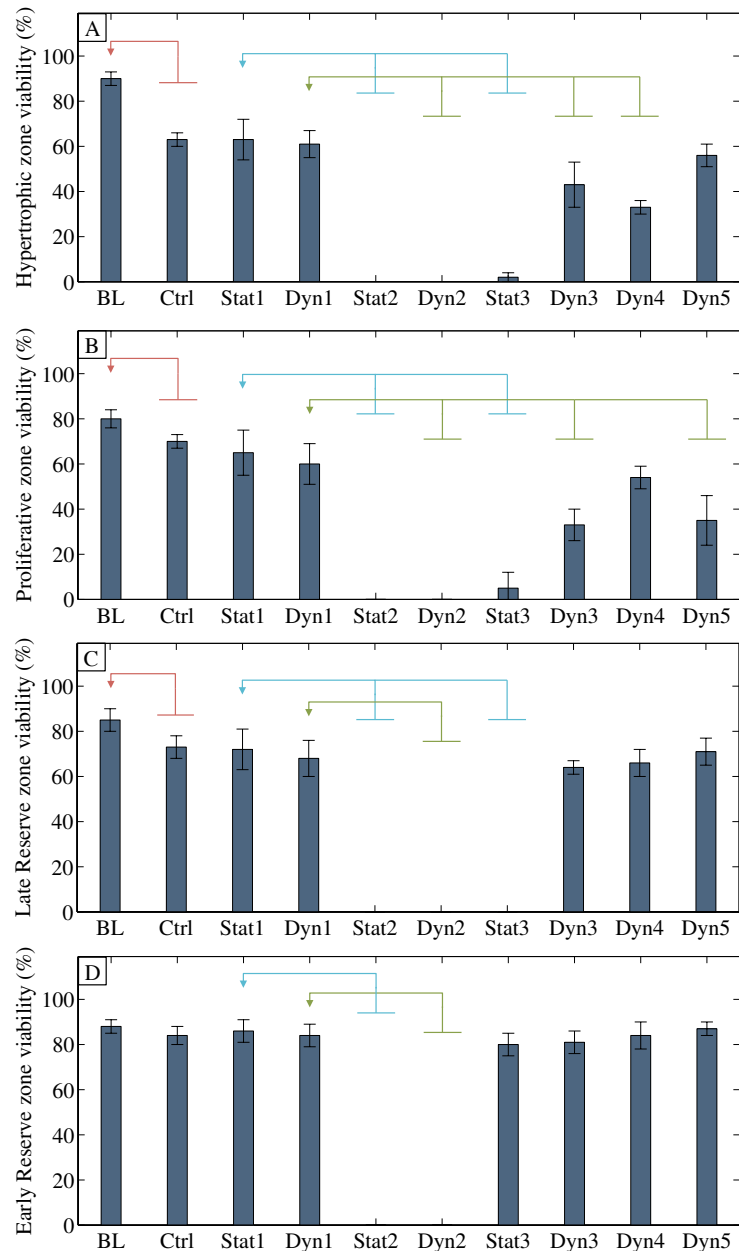
that of Dyn1 (Figure 3-7 A). No significant change was observed in the viability of the proliferative and of the two parts of reserve zone with respect to Dyn1 group (Figure 3-7 B-D)

Finally, dynamic modulation with 100% of amplitude around the average stress of 0.1 MPa for 12h/48 h (Dyn5) reduced the viability of proliferative chondrocytes to 58% of that of Dyn1 (Figure 3-7 B) while the viability of the hypertrophic, late and early reserve zones remained unchanged with respect to Dyn1 group (Figure 3-7 A, C-D).

Figure 3-7 Viability of different groups for each zone of growth plate.

A) Hypertrophic zone viability. B) Proliferative zone viability. C) Late reserve zone viability. D) Early reserve zone viability. Significant differences between groups ($p < 0.05$) are indicated with horizontal bars: red bars: significant difference between baseline and ctrl; blue bars: significant difference between different static groups; green bars: significant difference between different dynamic groups.

BL: Baseline, Ctrl (control), Stat1 (0.1 MPa, 12 hours), Stat2 (0.2 MPa, 12 hours), Stat3 (0.1 MPa, 24 hours), Dyn1 (0.1 MPa \pm 30%, 0.1 Hz, 12 hours), Dyn2 (0.2 MPa \pm 30%, 0.1 Hz, 12 hours), Dyn3 (0.1 MPa \pm 30%, 0.1 Hz, 24 hours), Dyn4 (0.1 MPa \pm 30%, 1.0 Hz, 12 hours), Dyn5 (0.1 MPa \pm 100%, 0.1 Hz, 12 hours)



3.6 Discussion

A priori knowledge on the effect of mechanical modulation parameters on growth plate explants cellular viability is a prerequisite for further investigations and precise interpretation of the effects of mechanical modulation on the biomechanics and biological responses of the tissue. The present study was the first to systematically investigate the effect of mode, magnitude, frequency, amplitude and duration of finely controlled compressive stress on the viability of growth plate chondrocytes following *in vitro* explants modulation.

Growth plate explant culture affects the viability of proliferative and hypertrophic chondrocytes.

The hypertrophic zone was found to be the most vulnerable zone to the culturing induced cell death with 30% of reduction in viability. In the proliferative zone and late reserve zone, 13% and 14 % of reduction in viability was observed respectively while the early reserve zone viability was not affected by culturing. Compared to the growth plate, the viability of articular cartilage chondrocytes was shown to be unaffected by culturing for durations as long as 14 days (Dumont, Ionescu et al. 1999, Strehl, Tallheden et al. 2005). This corroborates with our results for the growth plate reserve zone, which is most similar to articular cartilage in structure and composition. Chondrocytes of this zone are in a relatively latent state and hence their viability might be less sensitive to environmental changes. On the contrary, hypertrophic and proliferative chondrocytes are respectively actively involved in cellular division and volume enlargement as part of the growth process under specific signaling pathways, such as the PTH/PTHrP feedback loop for example (St-Jacques, Hammerschmidt et al. 1999). This interpretation is however limited because the reduction in viability could also be related to an impaired diffusion of the culture medium throughout the cultured explants.

Static and moderate dynamic modulation with low magnitude and duration maintain chondrocytes viability.

Based on preliminary studies, the preload of 0.001 N during 48 hours of culturing did not change the viability of chondrocytes in different zones of growth plate with respect to cultured samples without any preload (data not shown). Also, static and dynamic modulation (0.1 Hz, 30% amplitude) with low average stress applied for 12 hours did not significantly affect the viability

of the four zones of growth plate explants with respect to the control group. This suggests that both these compressive modulations remained within physiological limits tolerable by the tissue. However, increasing the average stress (to 0.2 MPa) reduced drastically the viability of all the zones. Similarly, in articular cartilage the viability is reduced in response to increment of stress (Chen, Bhargava et al. 2003, Torzilli, Deng et al. 2006). However, in articular cartilage, only the increment of stress from 0.1 MPa to 1.0 MPa or 5 MPa resulted in a similar result as we increased the stress from 0.1 MPa to 0.2 MPa in the growth plate. Differences between growth plate chondrocytes and articular cartilage chondrocytes might explain these observations. Growth plate is a very dynamic and biologically active tissue with cells that continuously proliferate, mature and hypertrophy, which could make them more sensitive to their physicochemical environment. However, in articular cartilage cells are in a more quiescent state, which could make them less responsive to the changes in their surrounding environment. Moreover, the extracellular matrix biocomposition is different in growth plate and cartilage and as a result the deformation pattern of the two tissues under compressive stress and the amount of load that is transmitted to the chondrocytes are not the same. In growth plate, the mechanical response of the tissue under compressive loading is zone dependent (Sergerie, Lacoursiere et al. 2009, Amini, Mortazavi et al. 2013) and in the hypertrophic and proliferative zones, the modulus of elasticity is almost half of its value than in the reserve zone (Sergerie, Lacoursiere et al. 2009) and articular cartilage (Mow and Ratcliffe 2005). As a result, these two zones undergo higher deformation and transmit higher mechanical stimulus to the chondrocytes of these two zones. This finding is consistent with an *in vivo* study on growth plate of three animal species by Stokes et al., which showed that growth rate is reduced in response to increased compression (Stokes, Aronsson et al. 2006). One limit of our experiment is that the effect of increasing the magnitude has been tested only with one combination of frequency and amplitude for the dynamic group. By lowering the frequency or/and magnitude, there might exist combinations of mechanical parameters maintaining chondrocyte viability.

Chondrocytes viability is duration dependent.

Increasing the modulation duration from 12h to 24h (Stat1 to Stat3, and Dyn1 to Dyn3) reduced the viability both in static and dynamic groups. This is similar to the response of articular cartilage to increased loading duration (Lucchinetti, Adams et al. 2002, Chen, Bhargava et al. 2003, Levin, Chen et al. 2005) and indicates that even non-damaging combinations of mechanical

parameters can become damaging both in static and dynamic modes when applied for a longer period of time. Considering that the viability of static group was reduced to a greater extent compared to the dynamic group (96% vs. 29% for the hypertrophic zone and 92% vs. 45% for the proliferative zone), it is suggested that static loading could be more detrimental than dynamic loading when sustained for longer modulation duration. A similar response was observed in articular cartilage, where chondrocytes death started sooner in response to static vs. dynamic modulation (Lucchinetti, Adams et al. 2002). This might be because in static modulation, net fluid exudation from the matrix is sustained for a longer period of time while dynamic modulation facilitates fluid exchange. This lack of fluid exchange could then be associated quicker cell death in static modulation. Also, compared to static loading, dynamic loading increases and facilitates nutrients transport in the culture medium (Mauck, Hung et al. 2003, Albro, Chahine et al. 2008), which could also be associated with an enhanced effect on cell viability in dynamic samples.

Increasing the frequency or amplitude of dynamic modulation has different effects on the viability of chondrocytes in different zones.

Increasing the frequency of dynamic modulation decreased the viability of chondrocytes in the hypertrophic zone while increasing the amplitude of loading decreased the viability of proliferative chondrocytes. The underlying factors involved in these observed changes remain to be determined. In parallel with our results, no frequency (Sauerland, Raiss et al. 2003) related effect was observed on the viability of chondrocytes in articular cartilage. This somehow corroborates with our results, as no changes were observed in the reserve zone cell viability, which remained similar for all dynamic cases. To the best of our knowledge to date, no study has addressed the effect of dynamic loading frequency and amplitude on hypertrophic and proliferative cell viability of growth plate.

Chondrocytes viability is zone dependent within the growth plate

Similar to articular cartilage (Chen, Bhargava et al. 2003), the viability of chondrocytes in growth plate is zone dependent. In most of the loading configurations, the hypertrophic zone was shown to be the most vulnerable zone to the mechanical loading while the reserve zone was shown to be the least sensitive to loading. This could be explained by respective mechanical properties, which are based on different extracellular matrix biocomposition. Under compressive loading, the

hypertrophic and proliferative zones undergo higher deformation and, as a result, the cells are more stimulated in these two zones. Conversely, deformation of the tissue is greatly reduced in the reserve zone, which is approximately two times more rigid than the H and P zones (Sergeie, Lacoursiere et al. 2009, Amini, Mortazavi et al. 2013), and reserve chondrocytes are consequently less disturbed. This combined with the lack of signalling in *in vitro* setting, which is essential for cell proliferation and hypertrophy, could explain the higher cell death proportion in these two zones.

3.7 Conclusion

This study, documented that chondrocytes viability in *in vitro* mechanically modulated growth plate is magnitude, frequency, amplitude, duration and zone dependent. In response to increment of magnitude, duration, frequency and amplitude, the viability of different growth plate zones was affected. Results also suggest that a longer duration under static loading might be more detrimental than under cyclic loading. However, more investigation is required on the effects of mechanical modulation parameters on biomechanical properties and protein expression of growth plate and on the possible relationships between chondrocyte viability and growth plate biomechanics and biology. Also, the mechanism of cell death in response to each set of parameters needs to be further investigated. The results of this study will be helpful in developing an *in vitro* protocol for studying the effects of mechanical modulation on growth plate biomechanical and biological responses. In long term, this knowledge will help finding the efficient and non-detrimental parameters for the mechanical modulation of bone growth, which is the basis for development of new fusionless approaches for the treatment of moderate scoliosis.

3.8 Acknowledgement

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CHAPTER 4 ARTICLE #2 GROWTH PLATE CARTILAGE SHOWS DIFFERENT STRAIN PATTERNS IN RESPONSE TO STATIC VERSUS DYNAMIC MECHANICAL MODULATION

This chapter introduces the second article written in the context of this thesis and responds to the second and third objectives of this thesis as detailed in Chapter 2.

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The contribution of the first author in the preparation, obtaining the results, writing and literature review of this paper is estimated at 85%.

Contribution of all authors:

Rosa Kaviani: Design of experiment, obtaining the results, analysis, interpretation of results, article writing and editing, responsible for the integrity of the work.

Irene Londono: Technical help

Stefan Parent: Design of experiment, Review of the article.

Florina Moldovan: Design of experiment, interpretation of results, review of the article, responsible for the integrity of the work.

Isabelle Villemure: Design of experiment, interpretation of results, review of the article, responsible for the integrity of the work.

4.1 Abstract

Longitudinal growth of long bones and vertebrae occurs in growth plate cartilage. This process is partly regulated by mechanical forces, which are one of the underlying reasons for progression of progressive growth deformities such as idiopathic adolescent scoliosis and early onset scoliosis. This concept of mechanical modulation of bone growth is also exploited in the development of fusionless treatments of these deformities. However, the optimal loading condition for the mechanical modulation of growth plate remains to be identified. The objective of this study was to evaluate the effects of *in vitro* static vs. dynamic modulation and of dynamic loading parameters, such as frequency and amplitude, on the mechanical responses and histomorphology of growth plate explants. Growth plate explants from distal ulnae of 4-week-old swines were extracted and randomly distributed among six experimental groups: baseline (n=10), control (n=10), static (n=10) and dynamic (n=3 × 10). For static and dynamic groups mechanical modulation was performed *in vitro* using an indexed cartigen bioreactor. A stress relaxation test combined with confocal microscopy and digital image correlation was used to characterize the mechanical responses of each explant in terms of peak stress, equilibrium stress, equilibrium modulus of elasticity and strain pattern. Histomorphometrical measurements were performed on toluidine blue tissue sections using a semi-automatic custom developed Matlab toolbox. Results suggest that compared to dynamic modulation, static modulation changes the strain pattern of the tissue and thus is more detrimental for tissue biomechanics while the histomorphological parameters are not affected by mechanical modulation. Also, under dynamic modulation, changing the frequency or amplitude does not affect the biomechanical response of the tissue. Results of this study will be useful in finding optimal and non-damaging parameters for the mechanical modulation of growth plate in fusionless treatments.

4.2 Keywords

Growth plate, Mechanical Modulation, Static Modulation, Dynamic Modulation, Mechanical Characterization, Digital Image Correlation, Histomorphometry

4.3 Introduction

The altered mechanical environment around the growth plate cartilage is known to be one of the modulating factors that can affect the growth rate of bones in children and adolescents (Stokes, Spence et al. 1996, CANCEL, Grimard et al. 2009, Valteau, Grimard et al. 2011). This phenomenon known as the mechanical modulation of bone growth is considered to be the underlying cause of progression of developmental growth abnormalities such as scoliosis (Stokes, Spence et al. 1996)

Among the various treatment options for scoliosis, the newly emerging minimally invasive fusionless approaches are considered to have fewer disadvantages for early onset of scoliosis or for moderate adolescent idiopathic scoliosis, when the patient still has growth potential (Guille, D'Andrea et al. 2007, Gomez, Lee et al. 2011). In comparison to conventional treatments using fusion of vertebrae, these types of treatments do not prevent the vertebral growth and they rely on remaining growth potential to correct the deformity. Also, no final fusion of vertebrae, which would lead to limited spinal mobility, is needed with these treatments (Aronsson and Stokes 2011).

Growth of long bones and vertebrae occurs in the growth plates located at the extremities of long bones and vertebrae (Ballock and O'Keefe 2003). This cartilaginous tissue is composed of chondrocytes, surrounded by an extracellular matrix. The chondrocytes are found in three different stages of differentiation forming three distinct zones: reserve (R), proliferative (P) and hypertrophic (H) (Ballock and O'Keefe 2003). The extracellular matrix is mainly composed of large aggregating proteoglycans (principally aggrecan) embedded within type II collagen fibrils (Mwale, Tchetina et al. 2002, Ballock and O'Keefe 2003). These two components are believed to be the critical determinants of the growth plate biomechanical behavior in compression (Amini, Mortazavi et al. 2012).

Although fusionless treatments have been evaluated in animal studies (Schmid, Aubin et al. 2008) and some clinical cases (Betz, D'Andrea et al. 2005), the optimal loading parameters and the effect of different mechanical modulation parameters on the growth plate has not been fully characterized.

Many studies have previously studied the effect of compression regimen (static and/or dynamic), magnitude, frequency and amplitude of dynamic modulation on bone growth rate (Cancel, Grimard et al. 2009, Valteau, Grimard et al. 2011, Ménard, Grimard et al. 2014), vertebral wedge angles (Akyuz, Braun et al. 2006), histology (Sergerie, Parent et al. 2011, Valteau, Grimard et al.

2011, Ménard, Grimard et al. 2014), *in vitro* viability (Kaviani, Londono et al. 2015) and protein expression (Cancel, Grimard et al. 2009, Sergerie, Parent et al. 2011) of the growth plate.

However, still the exact effect of mechanical modulation parameters on possible changes in the biomechanical behaviour of the growth plate tissue, which could consequently affect its biological function, has not been investigated.

Several *in vitro* studies have previously addressed the characterization of the intrinsic mechanical properties of growth plate tissue in non-pathological animal models. Using a transversely isotropic biphasic model (Cohen, Lai et al. 1998, Wosu, Sergerie et al. 2012), the bulk mechanical properties of the growth plate have been evaluated with an unconfined compressive stress relaxation tests. However, as shown in the studies by (Villemure, Cloutier et al. 2007, Sergerie, Lacoursiere et al. 2009) and (Amini, Mortazavi et al. 2012), mechanical properties of the growth plate are non-uniform throughout its three consecutive zones and they are in direct relationship with the biocomposition of the three growth plate zones (Amini, Mortazavi et al. 2012). In the two studies by (Villemure, Cloutier et al. 2007) and (Amini, Mortazavi et al. 2012), a combination of confocal microscopy, mechanical loading and digital image correlation was used for zone-wise characterization of mechanical properties of the growth plate. Both studies reported a zone-dependent strain pattern throughout the growth plate thickness (Villemure and Stokes 2009, Amini, Mortazavi et al. 2012).

To date, however, no study has investigated the effect of mechanical modulation parameters on possible changes in the deformational patterns of growth plate throughout its different zones. The objective of this study was to evaluate the effect of compression regimen (static vs. dynamic), frequency and amplitude of dynamic modulation on the growth plate biomechanical properties and histomorphometry. Results of this study will provide an improved understanding of the mechanisms of bone growth modulation. This knowledge will in turn be useful for the improvement and development of new fusionless treatment approaches using local modulation of growth for the correction of deformities in early onset of scoliosis or moderate adolescent idiopathic scoliosis.

4.4 Methods

4.4.1 Animal Model, Sample Preparation and Culture Systems

Ulnae of 4-week old swine were obtained from a local abattoir within 2 hours of slaughter. This age was chosen in order to study the pubertal growth spurt period occurring around 28 days old. Considering that at biomechanical level, growth plates from different species and anatomical locations show similar behaviour in response to compressive mechanical modulation, as shown by, porcine ulnar growth plates were used in this study for their size, relatively straight profile and ease of access. Explants composed of diaphyseal bone, growth plate and metaphyseal bone (total height of 9 mm) were extracted aseptically from distal ulnae using a 6 mm biopsy punch (Figure 4-1 A). The upper and lower surfaces were trimmed using a vibratome (Vibratome1500 Sectioning System) to obtain two parallel surfaces. The samples were randomly distributed among six different experimental groups: baseline ($n=1\times 10$); control ($n=1\times 10$); static ($n=1\times 10$) and dynamic ($n=3\times 10$) (Table 4-1). Explants of the baseline group were tested right after dissection. Explants from all the other groups, were placed in chambers of an indexed CartiGen bioreactor (Tissue Growth Technology, Instron, Norwood, USA) (Figure 4-1 B). Samples were then incubated in a culture medium containing DMEM enriched with 20% heat deactivated FBS and 1% antibiotics (penicillin and streptomycin) at 37° in 95% humidity and 5% CO₂ for 48h hours. During the culture period, explants of static and dynamic group were modulated based on the protocol explained in section 4.4.2.

4.4.2 Mechanical Modulation

Mechanical modulation was performed using the Indexed Cartigen Bioreactor (Figure 4-1 B). A preload of 0.001 MPa (0.028 N) representing to the weight of the loading piston was applied on all cultured explants. Samples of the static and dynamic groups were further loaded along the direction of bone growth in radially unconfined compression using the bioreactor linear motor (resolution 0.01 mm) and the level of compression was controlled by the integrated load cell (resolution 0.01 N) (Figure 4-1 C).

The epiphyseal and metaphyseal parts of the explants were maintained attached on the explant to fulfill the role of physiological porous filter. The parameters used for the mechanical modulation of explants are summarized in Table 4-1. Mechanical modulation was performed for 12 hours

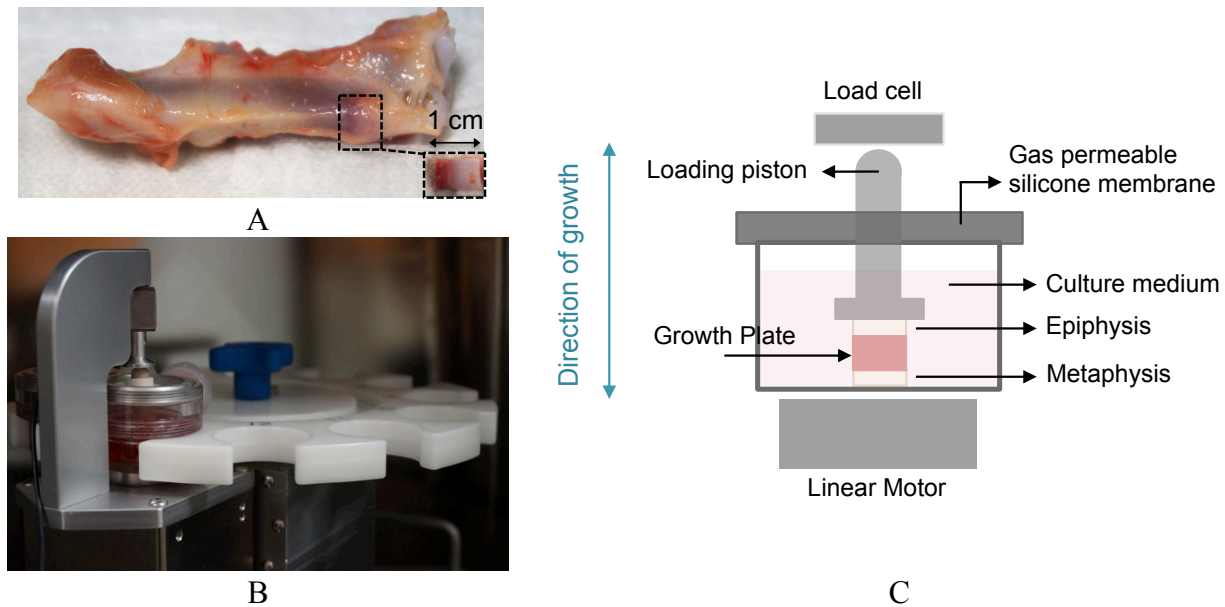


Figure 4-1 Growth plate sample and set up for mechanical modulation. A) Growth plate explant. B) Bioreactor. C) Bioreactor compression chamber

Table 4-1 Experimental groups and mechanical modulation parameters

Experimental Groups n = 10/group	Stress (MPa)	Dynamic Parameters	
		Amplitude (%)	Frequency (Hz)
Baseline		Fresh samples	
Control		Cultured for 48 hours	
Static	0.1	–	–
Dynamic			
Dyn1	0.1	30%	0.1
Dyn2	0.1	30%	1.0
Dyn3	0.1	100%	0.1

during the 48 hours of culture period intermittently (six repetitions of 2h loading, followed by 6 h of unloading). Static modulation was performed using an average stress of 0.1 MPa. Dynamic modulation was performed using a sinusoidal waveform with an average compressive stress of 0.1 MPa combined with: a) $\pm 30\%$ of average stress and 0.1 Hz of frequency (Dyn1), b) $\pm 30\%$ of average stress and 1.0 Hz of frequency (Dyn2) or c) $\pm 100\%$ of average stress and 0.1 Hz of frequency (Dyn3) (Figure 4-2).

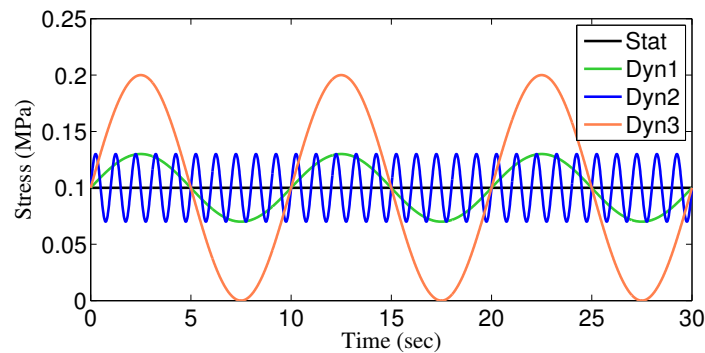


Figure 4-2 Static and dynamic loading protocols

4.4.3 Tissue Processing after Mechanical Modulation

Following mechanical modulation and culturing (or right after dissection for baseline samples), each sample was divided into two semi cylindrical halves along the direction of growth (Figure 4-3). One half was further divided and preserved with appropriate methods for biological assays. The other half was incubated in a 1M solution of Syto 17 (Molecular probes, Invitrogen, Montreal, Canada) to label chondrocytes nuclei for mechanical characterization of the tissue. After mechanical stress-relaxation test (Section 4.4.4), the semi cylindrical samples were embedded in methylmetacrylate (MMA) (Fisher Scientific Canada, Nepean, ON, Canada) for histomorphological studies.

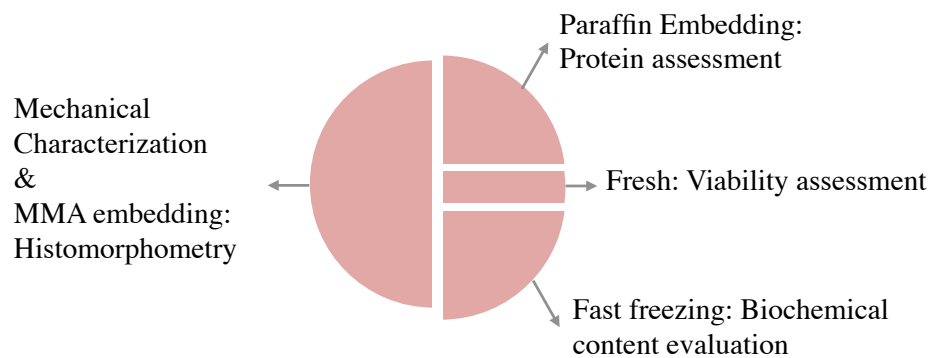


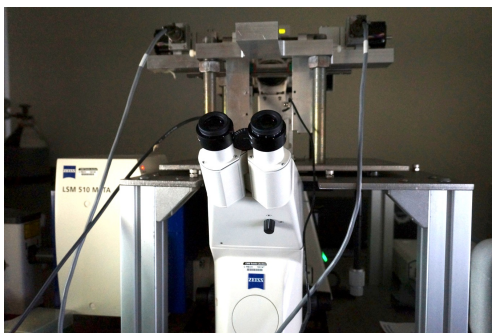
Figure 4-3 Explant partitioning for different tests

4.4.4 Mechanical Characterization and Confocal Imaging

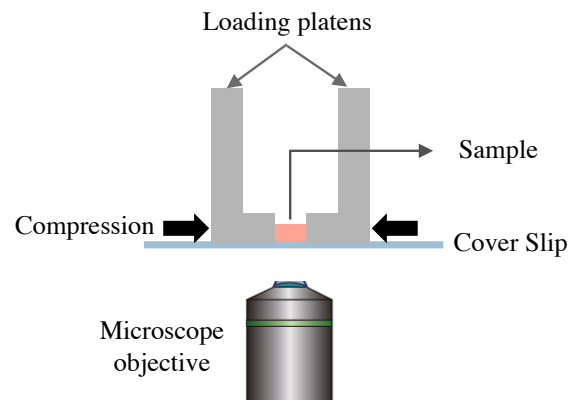
Similar to the study of Amini et al. 2013 (Amini, Mortazavi et al. 2012), mechanical characterization was performed through a semi confined stress-relaxation test using a custom made mechanical testing machine mounted on the stage of an LSM 510 inverted confocal microscope (Figure 4-4 A). The stained semi-cylindrical samples were placed between the platens of the mechanical loading device and were positioned right above the microscope objective so that at the same time the images could be taken and the loading could be applied (Figure 4-4 B). Following a first contact between the platen and the sample (fixed at 0.1 N compressive stress), specimens were initially preloaded at 5% strain with a strain rate of $1.7\text{E-}3 \text{ s}^{-1}$ (Amini, Mortazavi et al. 2012). After relaxation, an image (1024×1024 pixels) of the fluorescently labeled chondrocyte nuclei was recorded using an EC Plan-Neofluar 5X/0.16 NA lens (Carl Zeiss Inc., Jena, Germany) with zoom of 1X from the central part of each sample and referred to as the reference image. The specimens were further loaded with 5% of strain and after relaxation a second image was taken referred to as the deformed image.

The relaxation criterion was fixed to $1\text{E-}6 \text{ N/s}$ similarly to the previous study by (Amini, Mortazavi et al. 2012).

During the whole stress relaxation procedure, samples were bathed in DMEM and forces were recorded using a load cell with resolution of 0.026 N. Platen displacements were controlled by a custom designed software (Lab View, National Instruments, Inc.,USA).



A



B

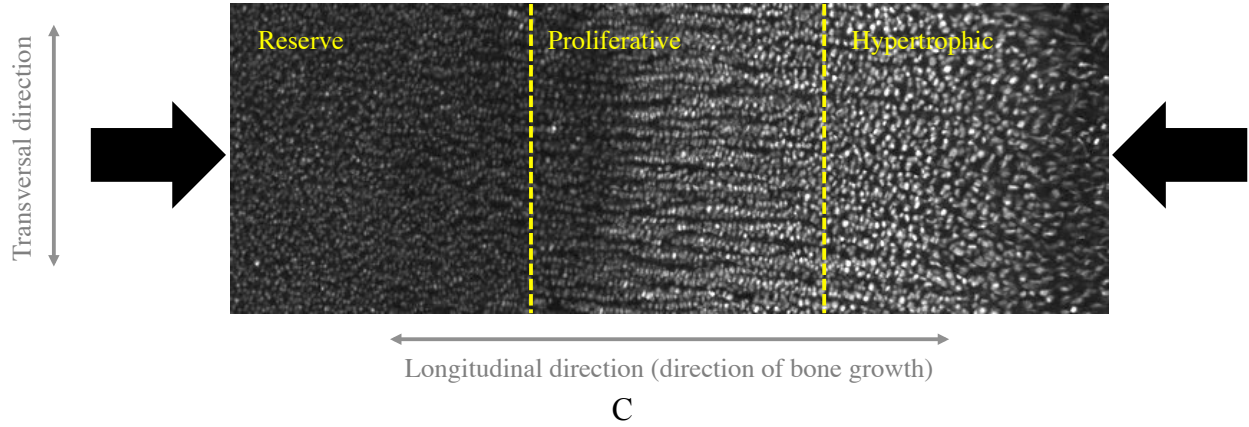


Figure 4-4 Mechanical characterization setup. A) Mechanical testing machine mounted on the stage of the inverted confocal microscope. B) Schematic of the sample placed in the mechanical characterization setup. C) Direction of application of the compressive stress

4.4.5 Digital Image Correlation

In the present study, similar to the study of (Amini, Mortazavi et al. 2013), displacements and strains were calculated using a full-field speckle pattern digital image correlation algorithm (Cheng, Sutton et al. 2002). For each sample, a region of interest (ROI) with the size of 800×400 pixels, spanning the three zones of the growth plate, was chosen in the reference and deformed images. The goal of the DIC algorithm is to build a spatial correlation between the reference image and the deformed image by minimizing the dissimilarity criterion function defined in Equation 4-1.

$$C = \sum_i \sum_j \left[\frac{I^{ref}(x_i, y_j) - I_0^{ref}}{\Delta I^{ref}} - \frac{I^{def}(x_i, y_j) - I_0^{def}}{\Delta I^{def}} \right],$$

$$I_0 = \frac{1}{M \times N} \sum_{i,j} I^{def}(x_i, y_j),$$

$$\Delta I = \sqrt{\sum_{i,j} (I(x_i, y_j) - I_0)^2}$$

Equation 4-1

where I_{ref} and I_{def} are the reference and deformed image intensity matrices. $M \times N$ is the size of the ROI. The displacement (u, v) is calculated by the difference between the coordinates of the pixel in the reference (x, y) and deformed images (\bar{x}, \bar{y}) (Equation 4-2).

$$\begin{aligned} u &= \bar{x} - x \\ v &= \bar{y} - y \end{aligned} \quad \text{Equation 4-2}$$

In our algorithm, the B-Spline function is used to represent the continuous deformation field throughout the image. Therefore, the objective of the algorithm was to find the best B-Spline parameters for minimum dissimilarity between the two images. The algorithm was implemented in MATLAB (2014a, The MathWorks, Inc., Natick, Massachusetts, United States).

4.4.6 Calculation of Mechanical Responses

The peak and equilibrium stresses were extracted from the recorded data by the sensor. The Lagrangian strain tensors in the longitudinal direction (Figure 4-4 C) were calculated by partial differentiation of displacement matrices. In order to calculate the strains per depth in each sample, the strain matrix was averaged in transversal direction (Figure 4-4 C). In order to calculate the bulk strain in each growth plate zone, the strain matrix was averaged throughout that zone. Furthermore, for more representative comparison between experimental groups, the strains were normalized to the thickness of each growth plate zones resulting in strain per percentage of thickness of each zone. Finally, the modulus of elasticity at equilibrium state was calculated by dividing the equilibrium stress by the calculated total strain of the tissue.

4.4.7 Histomorphology

For quantification of the thickness of the three growth plate zones, 5 μm sections of the MMA embedded tissue were cut using a rotary microtome (Leica RM 2145) along the longitudinal direction. For each sample, three sections chosen from three different depths in the explant were stained with toluidine blue. Digital images of the complete growth plate sections were taken using an optical microscope (Leica DMR) equipped with Retiga Qimaging Camera with 10% magnification. Similar to previous studies (Valteau, Grimard et al. 2011, Ménard, Grimard et al. 2014), the thickness evaluation for the three histological zones (reserve, proliferative and hypertrophic) was performed using a semi-manual custom developed MATLAB toolbox. At first, the borders of the three zones were manually selected on each image based on the visual differences in the shape, size and arrangement of chondrocytes in the three different zones. The boundary between the reserve and the proliferative zones was identified by the upper margins of the proliferative columnar arrangement and the boundary between proliferative and hypertrophic

zones was identified by the first chondrocytes that had a significantly increased size relative to the proliferative cells. Then, the thickness of each zone was calculated by averaging the distance between its upper and its lower borders (Figure 4-5). The thickness of each zone was then normalized to the thickness of the growth plate. This procedure was repeated on three sections for each sample and the average value was reported as the thickness of each zone in each sample.

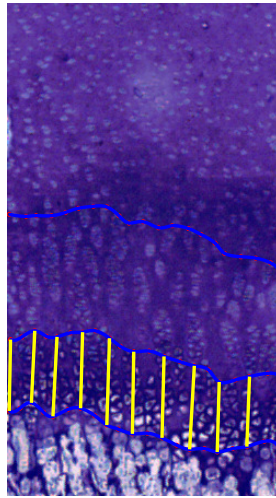


Figure 4-5 Histomorphological analyses: example of the procedure for evaluating the thickness of the hypertrophic zone

4.4.8 Statistical Analysis

Basic statistical analysis (mean, SD) was performed on the thicknesses of growth plate zones, peak stress, equilibrium stress and equilibrium modulus of elasticity. To determine the effect of different modulation parameters on the mentioned parameters, a one-way ANOVA followed by Tukeys post-hoc comparisons were performed. Also, in order to identify the significant differences between the strain patterns in different groups, the normalized strain per percentage of depth were averaged in each group and the confidence interval was calculated. Afterwhile, the average strain per percentage of depth of the six groups of experiment and their confidence intervals were plotted and qualitatively compared.

4.5 Results

4.5.1 Comparison of the Bulk Mechanical Responses

The average and standard deviation of peak stress (σ_p), equilibrium stress (σ_e), calculated equilibrium modulus of elasticity (E_e) of the complete growth plate as well as the strains in the hypertrophic (ϵ_H), proliferative (ϵ_P) and reserve (ϵ_R) zones are summarized in Table 4-2 and presented in Figure 4-6.

Statistically significant decrements were found in the peak stresses (Figure 4-6 A), equilibrium stresses (Figure 4-6 B) and moduli of elasticity (Figure 4-6 C) of all cultured groups compared to baselines. In the hypertrophic zone, the bulk strains were significantly increased in the static group with respect to controls (Figure 4-6 D).

In the proliferative zone, the bulk strains of all cultured groups remained unchanged compared to baseline group (Figure 4-6 D) while, in the reserve zone, all the cultured groups showed significantly lower strains than baselines (Figure 4-6 D).

Table 4-2 Peak stress (σ_p), equilibrium stress (σ_e), equilibrium modulus of elasticity (E_e) and strain of hypertrophic (ϵ_H), proliferative (ϵ_P) and reserve (ϵ_R) zones (significant differences $p < 0.05$ with respect to baseline and control groups are shown with * and ** respectively)

Groups	σ_p (MPa)	σ_e (MPa)	E_e (MPa)	ϵ_R	ϵ_P	ϵ_H
Baseline	0.142 ± 0.016	0.098 ± 0.016	2.031 ± 0.192	1.208 ± 0.461	1.249 ± 0.444	0.948 ± 0.332
Control	$0.044 \pm 0.010^*$	$0.024 \pm 0.005^*$	$0.504 \pm 0.078^*$	$0.366 \pm 0.201^*$	1.730 ± 0.205	1.154 ± 0.240
Static	$0.037 \pm 0.037^*$	$0.026 \pm 0.007^*$	$0.537 \pm 0.146^*$	$0.255 \pm 0.204^*$	1.453 ± 0.290	$1.853 \pm 0.255^{**}$
Dynamic						
Dyn1	$0.054 \pm 0.010^*$	$0.028 \pm 0.006^*$	$0.556 \pm 0.106^*$	$0.371 \pm 0.172^*$	1.821 ± 0.213	1.552 ± 0.160
Dyn2	$0.048 \pm 0.009^*$	$0.028 \pm 0.006^*$	$0.559 \pm 0.132^*$	$0.434 \pm 0.235^*$	1.645 ± 0.162	1.378 ± 0.257
Dyn3	$0.038 \pm 0.007^*$	$0.021 \pm 0.001^*$	$0.420 \pm 0.041^*$	$0.411 \pm 0.051^*$	1.685 ± 0.273	1.023 ± 0.265

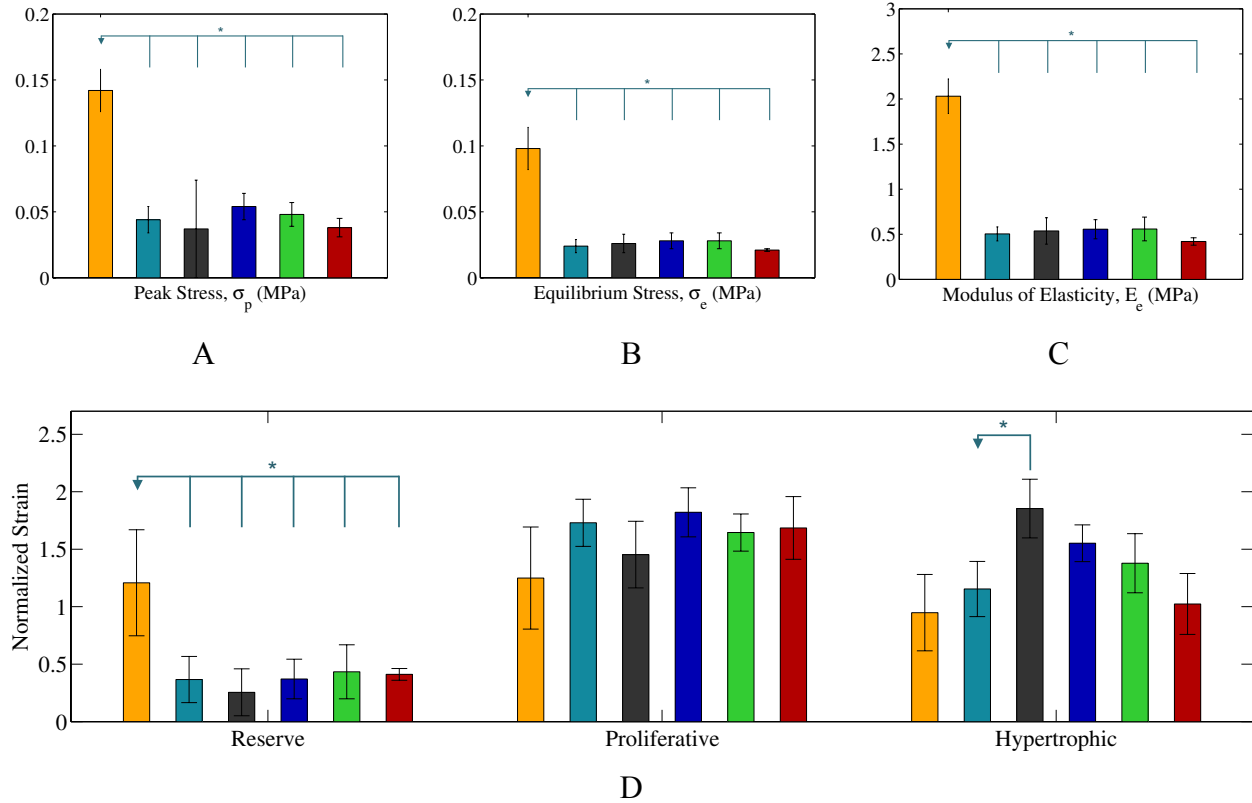


Figure 4-6 Mechanical parameters of complete growth plate and normalized zonal strain (significant differences ($p < 0.05$) marked with *)

4.5.2 Comparison of Strain Patterns through Growth Plate Thickness

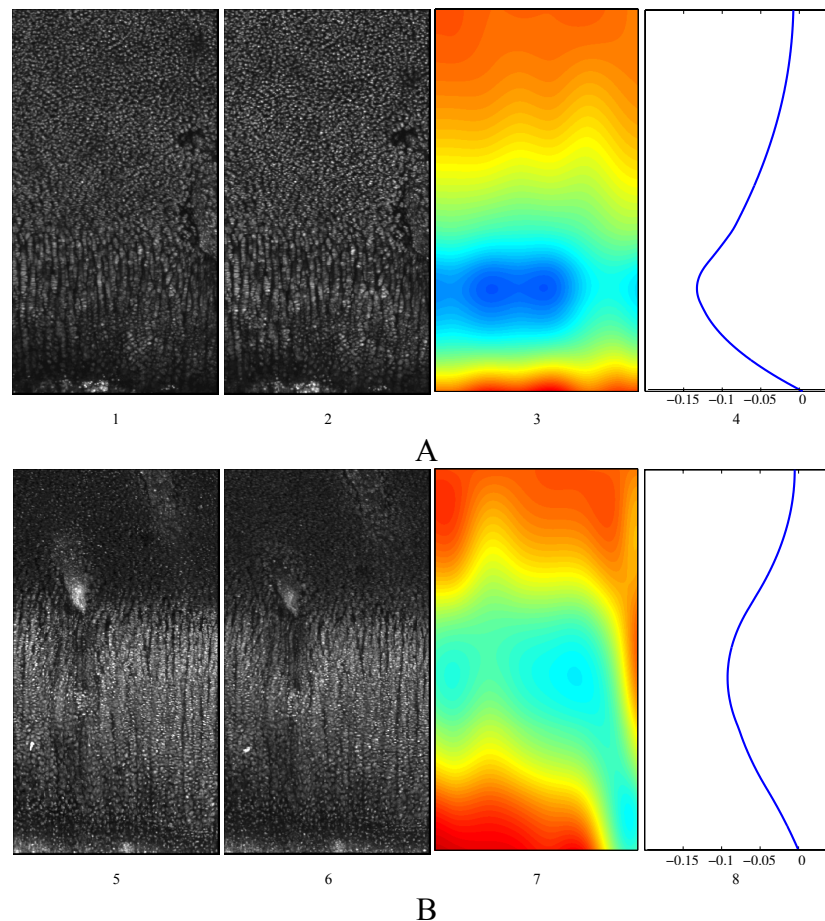
Figure 4-7 shows representative strain patterns along growth direction for different groups. As it can be observed, samples from all groups show a zone dependent strain pattern with higher strain in the proliferative zone for baseline and control groups, in the intersection of proliferative and hypertrophic zones for the three dynamic groups and in the hypertrophic zone for the static group. By superimposing the average normalized strain per percentage of depth graphs and their confidence intervals for the different groups, the depth dependent differences in the strain pattern of different zone can be extracted more clearly (Figure 4-8). In order to isolate the culturing effect, normalized strains of baseline and control groups were compared: no significant difference was observed between strain patterns of the three growth plate zones (Figure 4-8 A).

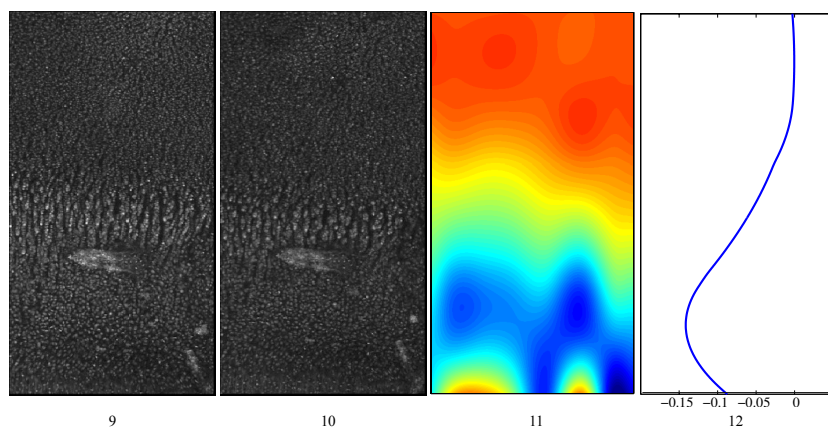
In order to compare static and dynamic modulation, Stat and Dyn1 groups were compared with controls (Figure 4-8 B). In the static group, strains of the hypertrophic zone were significantly

increased and strains of the proliferative and reserve zones were significantly decreased with respect to the control group. A similar trend as for the static group was observed for the moderate dynamic group (Dyn1), the strain in the hypertrophic zone was higher than control, although not significant, and in the proliferative zone, the strain was significantly lower than controls. In the reserve zone, the strain was not significantly decreased compared to controls.

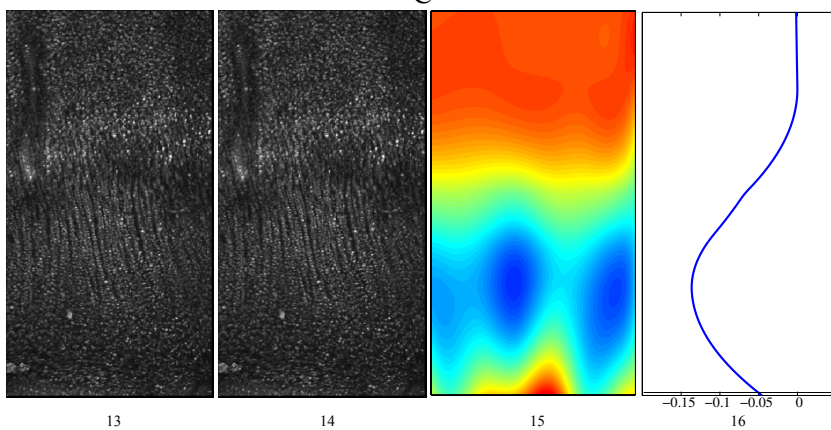
When comparing static vs. dynamic modulation (Figure 4-8 B), the strain was significantly higher in the proliferative zone and at the intersection of proliferative and hypertrophic zones of the dynamic group while it was lower in the hypertrophic zone.

In order to evaluate the effects of dynamic loading parameters, the three dynamic groups (Dyn1, Dyn2, Dyn3) were compared. No significant difference was observed when changing either the frequency (Dyn2 vs. Dyn1) or changing the amplitude (Dyn3 vs. Dyn1) (Figure 4-8 C).

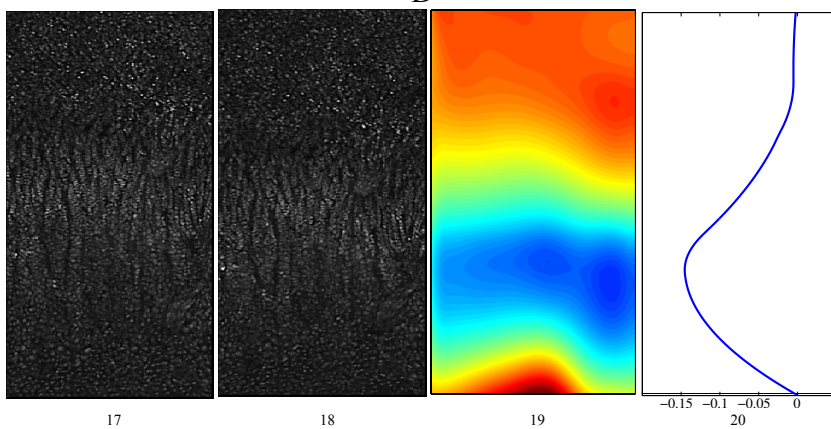




C



D



E

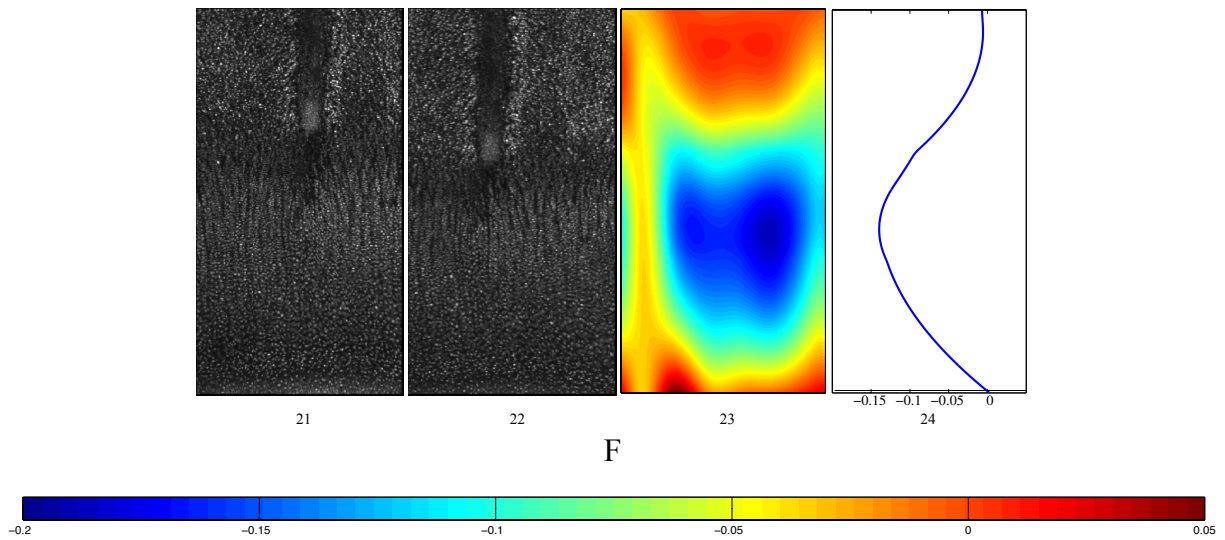


Figure 4-7 Representative strain patterns obtained for each experimental group. First and second columns on the left show reference (1, 5, 9, 13, 17, 21) and deformed (2, 6, 10, 14, 18, 22) growth plate confocal images respectively. The third column from left (3, 7, 11, 15, 19, 23) shows the strain pattern within the growth plate thickness and the last column (4, 8, 12, 16, 20, 24) shows the resulting average strain throughout the thickness of the growth plate explant.

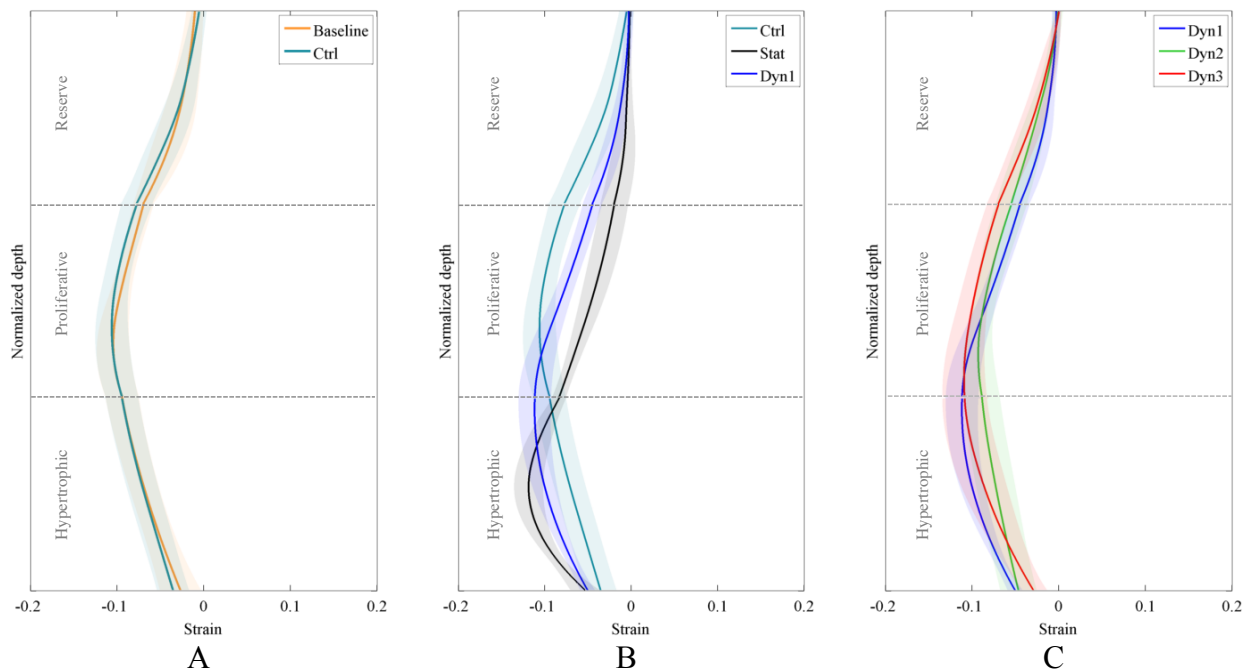


Figure 4-8 Average longitudinal strains normalized to the thickness of the three zones: A) effect of culturing, B) effect of static vs. dynamic compression, C) effect of dynamic loading parameters

4.5.3 Histomorphometry

Representative histological sections are shown for each group in Figure 4-9. Chondrocytes in the hypertrophic and proliferative zones were arranged in columns in the baseline and control groups (Figure 4-9 A, B). However, this columnar arrangement was lost in both the proliferative and hypertrophic zones of static modulated explants (Figure 4-9 C). In all the three groups of dynamic explants, the arrangement was preserved to some extent but the columns were deviated slightly from the longitudinal axis (Figure 4-9 D-F).

Results of histomorphological analyses in terms of average thicknesses of the three growth plate zones are summarized in Table 4-3 and Fig. Figure 4-10. No significant differences were observed between the thicknesses of the three zones among the six groups of experiment.

In order to reduce the effect of heterogeneity between different samples, the thickness of each zone was normalized with respect to total growth plate thickness. The normalized thickness for reserve, proliferative and hypertrophic zones are presented as percentage of total growth plate thickness in Table 4-3 in and in Figure 4-11. When comparing the baselines with all cultured groups, a significant increase was observed for the hypertrophic thickness percentage of the static and Dyn2 groups (Figure 4-11). The proliferative zone thickness percentage was also significantly increased in the dynamic group with 1.0 Hz frequency (Dyn1) compared to baselines (Figure 4-11).

Also, compared to baseline group, the reserve zone thickness was significantly decreased for static and Dyn1 groups (Figure 4-11).

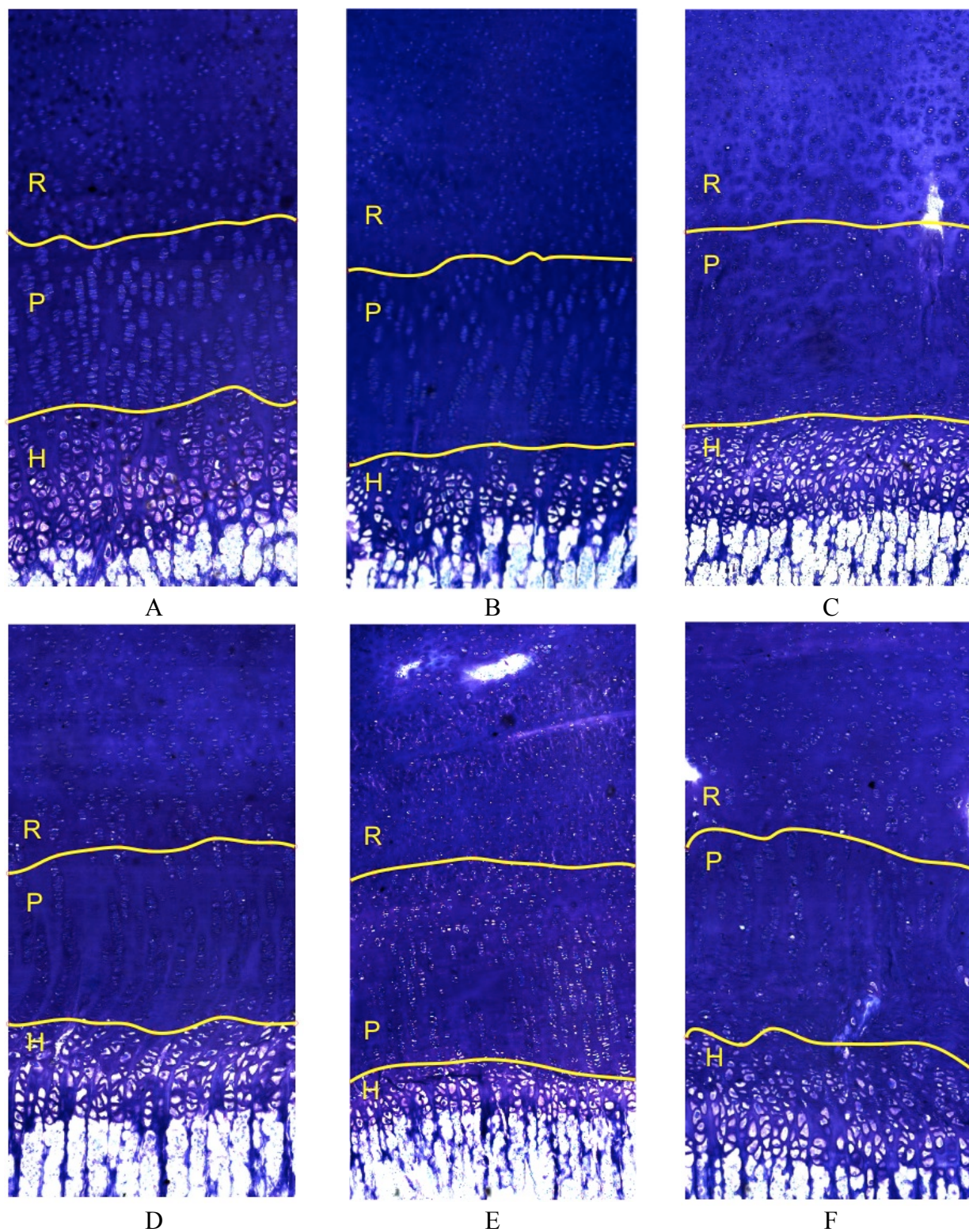


Figure 4-9 Representative toluidine blue stained growth plate sections for each experimental group. The borders of hypertrophic (H), proliferative (P) and reserve zones (R) are marked with yellow line

Table 4-3 Average thickness of hypertrophic, proliferative and reserve zones

Groups	Zonal Thickness (μm)		
	Reserve	Proliferative	Hypertrophic
Baseline	2249 \pm 300	514 \pm 55	308 \pm 95
Control	1782 \pm 214	465 \pm 127	249 \pm 64
Static	2091 \pm 393	541 \pm 89	328 \pm 78
Dynamic			
Dyn1	2005 \pm 342	554 \pm 67	280 \pm 71
Dyn2	1979 \pm 349	467 \pm 129	312 \pm 124
Dyn3	2249 \pm 300	514 \pm 55	308 \pm 95

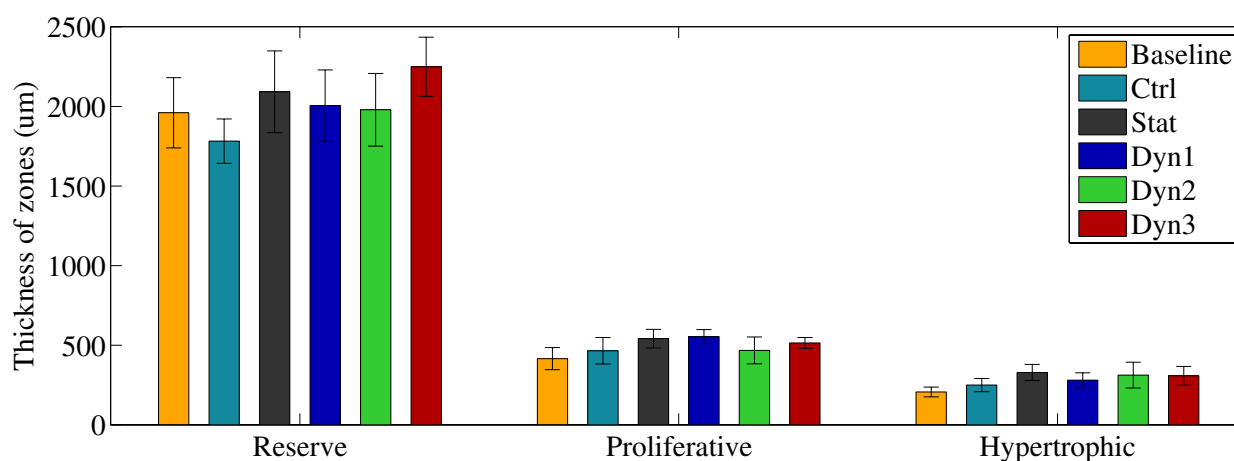


Figure 4-10 Average thicknesses of the three growth plate zones

Table 4-4 Average percentage of normalized thickness of hypertrophic, proliferative and reserve zones with respect to total growth plate thickness (significant differences in each zone with respect to baseline group are marked with * ($p < 0.05$))

Groups	Reserve	Proliferative	Hypertrophic
Baseline	76.02 \pm 2.81	16.02 \pm 2.18	7.96 \pm 0.94
Control	71.63 \pm 4.35	18.45 \pm 3.11	9.91 \pm 1.90
Static	70.46 \pm 3.14*	18.45 \pm 2.36	11.09 \pm 1.93*
Dynamic			
Dyn1	70.48 \pm 2.81*	19.69 \pm 2.20*	9.89 \pm 1.62
Dyn2	72.41 \pm 4.86	16.64 \pm 2.66	10.97 \pm 2.64*
Dyn3	3.49 \pm 5.53	16.59 \pm 2.65	9.92 \pm 3.53

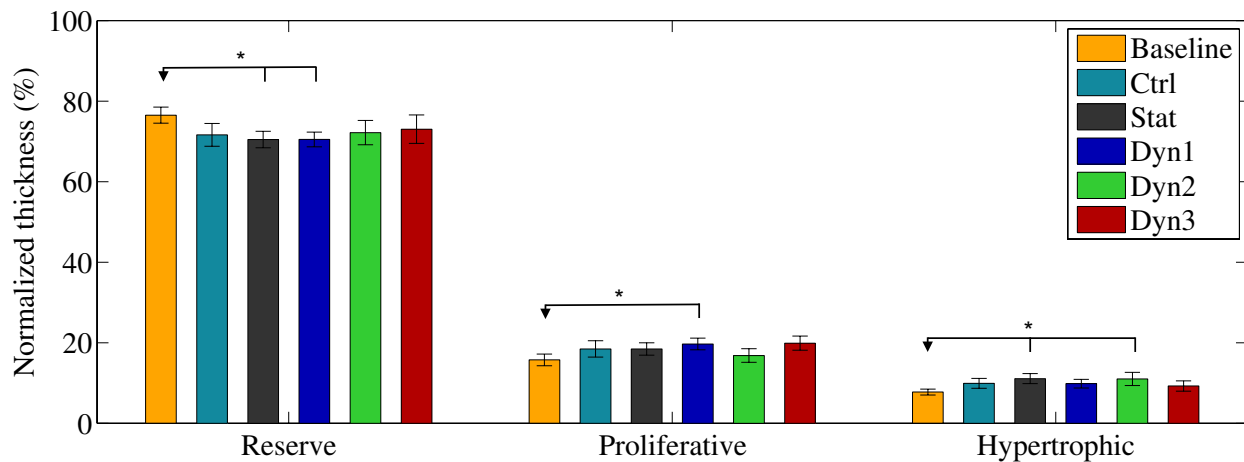


Figure 4-11 Average normalized thicknesses of the three growth plate zones (significant differences, $p < 0.05$, marked with *)

4.6 Discussion

The aim of this study was to determine the effects of *in vitro* compressive static versus dynamic modulation and dynamic loading parameters on local strain patterns of growth plate explants and on their histomorphometry. These results are greatly relevant for the improvement of new treatment approaches, which are based on the local modulation of vertebral growth to correct scoliotic deformities. In this study, for both mechanical modulation and mechanical characterization, unconfined compression was used to replicate the *in vivo* state of the tissue.

Under uniaxial compression, deformational patterns within growth plate tissue were found to be non-uniform and zone dependent in all groups. In the baseline group, similarly to a previous study by (Amini, Mortazavi et al. 2013), the higher axial strains were observed in the proliferative zone and the lower axial strains were observed in the reserve zone. Our results also corroborate with moduli of elasticity evaluated for the three zones of new born swine growth plates using quasi-static tests, where the reserve zone was found two times more rigid than the two other zones (Sergerie, Lacoursiere et al. 2009).

The equilibrium modulus of elasticity of the baseline samples 2.031 ± 0.192 MPa is similar to values reported for 4-week-old swine growth plates by (Ménard, Soullisse et al. 2014) 2.16 ± 0.56 MPa.

Culturing growth plate explants modifies their biomechanical properties. Indeed, the peak and equilibrium stresses of culture controls were reduced by 70% and 75% compared to baselines. The equilibrium modulus of elasticity was also found to be decreased by 75%. From a mechanical point of view, the mechanical behaviour of growth plate (similarly to articular cartilage) under unconfined compression can be described using a biphasic model such as the transversely isotropic biphasic model (Cohen, Lai et al. 1998) in which the growth plate is modeled as a mixture of a porous elastic isotropic solid and an inviscid incompressible fluid. In this model, the stress of the tissue is inversely related to the permeability of the solid matrix and thus decrement in the stress level of cultured samples could be partly described by an increased permeability of the solid matrix. An increased permeability can be associated with changes in the protein content (loss of proteoglycan or collagen) and/or organization (direction of collagen fibers) of components within the tissue (Armstrong and Mow 1982), which in turn facilitate the fluid flow inside the solid phase under unconfined compression. Changes in the protein content and organization can be a result of increased degradative enzymatic activity, loss of viability of chondrocytes or decreased micro-circulation which needs further investigations. However, the strain patterns of the culture control group throughout the thickness of the growth plate remained unchanged and similar to the baselines, with the higher axial strains observed within the proliferative zone and lower axial strains in the reserve zone. This suggests that although culturing reduces the modulus of elasticity and permeability of the tissue, it affects the three zones of growth plate similarly, so that the relative strain patterns remains unchanged.

Static modulation changes the deformational pattern within the growth plate compared to the control and dynamic groups. Compared to the control group, samples modulated with static loading had 60% more strain in the hypertrophic zone and a significantly lower strain in the proliferative and reserve zones. Since the mechanical responses of growth plate is correlated with its proteoglycan and collagen content as shown by Amini et al. 2013, this modified strain pattern within the static group could be attributed to protein content changes. As previously demonstrated in both growth plate (Sergerie, Parent et al. 2011) and articular cartilage (Grodzinsky, Levenston et al. 2000), aggrecan and collagen synthesis is inhibited in both tissues in response to static modulation and consequently the tissue becomes less rigid.

The bulk strain of samples modulated with moderate dynamic loading (Dyn1) was not significantly different from controls. However, in comparison with controls, Dyn1 samples had higher strains at the intersection between the hypertrophic and proliferative zones (not significant) and lower strains in the early proliferative and late reserve zones (significant). These observed strain patterns imply that similar changes as in static modulation occurred in the dynamic modulation but to a lower extent. These results corroborate with a recent study on the effect of static and dynamic compression on the viability of chondrocytes, which showed that short term dynamic compression as done in this study does not affect the viability of chondrocytes while long term dynamic compression decrease the viability (Kaviani, Londono et al. 2015) and might affect the extracellular matrix protein production. In growth plate, short term dynamic compression as opposed to static modulation was shown to better preserve the protein synthesis while in articular cartilage it was shown to have stimulating effects on protein synthesis in the tissue (Grodzinsky, Levenston et al. 2000, Sergerie, Parent et al. 2011); this might partly explain the preservation of mechanical responses in dynamic groups.

Different dynamic loading parameters do not impact the growth plate strain patterns. In this study, it was found that increasing the frequency from 0.1 Hz to 1.0 Hz or increasing the amplitude of loading from 30% to 100% does not affect strain patterns of the tissue.

These results correlate with the result of a finite element study, which showed that changing frequency and amplitude of dynamic loading do not have any significant effect on the stress components and deformation at the tissue level (Tireh-dast 2014). These results, also correlate with the results of an *in vivo* study by (Ménard, Grimard et al. 2014), where increasing the

compression frequency or amplitude did not have any effect on growth plate histomorphometry. Indeed, the bulk strains and the normalized strains of different growth plate zones were not significantly different between Dyn3 or Dyn2 with respect to Dyn1. This result is not in agreement with our previous *in vitro* study (Kaviani, Londono et al. 2015) on viability of chondrocytes. In that study, the viability of chondrocytes in the hypertrophic and proliferative zones was significantly decreased in response to increased amplitude or increased frequency respectively. Therefore, it was expected to observe a more pronounced change in the hypertrophic and proliferative zones strain in Dyn3 or Dyn2 group compared to Dyn1 because of possible changes in the protein content of the tissue. This discrepancy between zonal strains and chondrocyte viability in these zones might be partly described by the viscoelastic behaviour of growth plate. The total stress in the tissue is a balance between the stress in the solid matrix and the hydrostatic pressure of the fluid within the pores of extracellular matrix (Cohen, Lai et al. 1998). Therefore, the total stress applied to the chondrocytes is a combination of the stress transferred from the matrix and the hydrostatic pressure of the fluid. Using stress relaxation tests, only the deformation of the extracellular matrix could be evaluated while the hydrostatic pressure might be the reason for the loss of viability in high amplitude and high frequency groups. This would require further investigations.

Growth plate zonal height remained unchanged in response to compressive modulations. The thicknesses of the three zones of growth plate which can be correlated with growth rate of the bone (Wilsman, Leiferman et al. 1996) were similar in response to culturing and to both static and dynamic compressive modulations. In the baseline and culture control groups, the chondrocytes were arranged in columns in the direction of growth as expected for the normal state of the tissue (Burdan, Szumilo et al. 2009). However, in the static group, the columnar arrangement of chondrocytes was lost in the proliferative and hypertrophic zones while, in the dynamic groups, this columnar arrangement was preserved and the columns were only slightly deviated with respect to the growth direction. The loss of columnar arrangement of chondrocytes in the proliferative and hypertrophic zones of the static group might be an indicator of the loss of tissue integrity in these two zones, which would suggest that changes in the mechanical responses of statically modulated explants can be related to the changes in the extracellular matrix proteins (changes in protein content and/or organization).

Our results also correlate with the ones of (Sergerie, Parent et al. 2011), where it was observed that in response to both static and dynamic compression (using a higher average stress with approximate average of 0.2 MPa), the ratio of hypertrophic and proliferative zone to growth plate thickness remains unchanged. Although in their study, similar to ours, they observed a non-significant reduction in the proportion of hypertrophic and proliferative zones in the static group with respect to the controls. Our results are also in consistent with a recent *in vivo* study by (Ménard, Grimard et al. 2014) on the effect of dynamic loading parameters (using 0.2 MPa of average compression), where no differences were observed between the histomorphometrical parameters of growth plate samples modulated with two different frequencies and two different amplitudes.

In summary, this study allowed evaluating for the first time, the effects of static vs. dynamic compression and dynamic compressive parameters on the biomechanical response of growth plate tissue. Overall, results suggest that static modulation triggers more changes in the biomechanical responses of growth plate than dynamic modulation and disturbs the columnar arrangement of chondrocytes in the hypertrophic and proliferative zones. However, with the protocol used in this study, neither type of modulation had any effects on the thickness of different growth plate zones. Further investigations are needed to determine the possible changes in the extracellular matrix composition within the growth plate following the mechanical modulation. A future complementary study will be performed using the frozen parts and paraffin embedded parts of the explants to determine changes in the matrix composition.

Also, in the context of the protocol used in this study, frequency and amplitude of dynamic compressive modulation were found to not impact the biomechanical responses and the thickness of different growth plate zones.

Although extrapolation of these results to *in vivo* applications is limited, the obtained data will further our knowledge on the effects of mechanical modulation parameters and will be useful for development of effective and optimized fusionless implants for the treatment of scoliosis.

4.7 Acknowledgements

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CHAPTER 5 ARTICLE #3 CHANGES IN GROWTH PLATE EXTRACELLULAR MATRIX COMPOSITION AND BIOMECHANICS FOLLOWING IN VITRO STATIC VS. DYNAMIC MECHANICAL MODULATION.

This chapter introduces the third article written in the context of this thesis and responds to part of the fourth objective of this thesis as detailed in Chapter 2.

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The contribution of the first author in the preparation, obtaining the results, writing and literature review of this paper is estimated at 85%.

Contribution of all authors:

Rosa Kaviani: Design of experiment, obtaining the results, analysis, interpretation of results, article writing and editing, responsible for the integrity of the work.

Irene Londono: Technical help

Stefan Parent: Design of experiment, Review of the article.

Florina Moldovan: Design of experiment, interpretation of results, review of the article, responsible for the integrity of the work.

Isabelle Villemure: Design of experiment, interpretation of results, review of the article, responsible for the integrity of the work.

5.1 Abstract

Longitudinal growth, which occurs in cartilaginous growth plates, is highly regulated by mechanical forces and is the underlying progression factor of angular deformities such as scoliosis. The concept of mechanical modulation of bone growth is currently exploited for the development of fusionless treatments of scoliosis. However, efficient but non-damaging loading parameters are not well established. The objective of this study was to investigate the effects of mechanical modulation parameters on structural proteins biocomposition and mechanical properties of growth plate. The biocomposition was characterized using biochemical content evaluation and immunohistochemistry. Mechanical properties were characterized by fitting the data of the stress relaxation curves using a fibril reinforced biphasic model. Results showed that static loading triggers a decrease in proteoglycan content and type X collagen in specific zones of the growth plate. These changes can be associated with the observed decrement of permeability in the static group. Dynamic modulation did not affect the growth plate biocomposition and biomechanical responses. Results of this study provides an improved understanding of growth plate responses to its mechanical environment, which will be useful in finding the optimal and non-damaging parameters for fusionless treatments based on the mechanical modulation of bone growth.

5.2 Keywords

Growth plate, scoliosis, mechanobiology, mechanical modulation, compressive loading, quantitative immunohistochemistry, biochemical content evaluation.

5.3 Introduction

Longitudinal growth of long bones and vertebrae occurs in growth plate cartilage, located at their extremities (Ballock and O'Keefe 2003). Based on experimental and clinical evidences, mechanical forces are a key factor of longitudinal bone growth regulation and one of the main reasons for the progression of developmental growth angular deformities such as juvenile and adolescent scoliosis (Stokes, Spence et al. 1996). The concept of mechanical modulation of bone growth is currently used for the development of novel fusionless devices to correct spinal deformities (i.e. adolescent idiopathic scoliosis) by applying compression on the rapidly growing part of vertebrae located in the convex part of the curvature in order to retard growth (Guille, D'Andrea et al. 2007).

Experimental *in vivo* and *in vitro* studies are trying to find optimal but non damaging loading parameters for mechanically modulating bone growth by comparing the effects of static vs. dynamic loading (Sergerie, Parent et al. 2011, Valteau, Grimard et al. 2011, Kaviani, Londono et al. 2015, Kaviani, Londono et al. 2015) or comparing dynamic loading parameters (Ménard, Grimard et al. 2014, Kaviani, Londono et al. 2015, Kaviani, Londono et al. 2015) on bone growth rate (Cancel, Grimard et al. 2009, Valteau, Grimard et al. 2011, Ménard, Grimard et al. 2014), growth plate histomorphology (Cancel, Grimard et al. 2009, Sergerie, Parent et al. 2011, Valteau, Grimard et al. 2011, Ménard, Grimard et al. 2014, Kaviani, Londono et al. 2015), extracellular matrix protein synthesis (Cancel, Grimard et al. 2009, Sergerie, Parent et al. 2011), biomechanical responses (Kaviani, Londono et al. 2015) and chondrocyte viability (Kaviani, Londono et al. 2015). The overall results suggest that dynamic loading is less detrimental for tissue biomechanics and integrity while it has the same growth reducing effect as static loading. In our recent *in vitro* study (Kaviani, Londono et al. 2015), we also observed that static loading is more detrimental for tissue biomechanics as it changes the mechanical strain patterns within the growth plate tissue when compared to dynamic loading. Previous studies on growth plate mechanobiology from our group (Cancel, Grimard et al. 2009, Sergerie, Parent et al. 2011) and some studies on articular cartilage (Bachrach, Valhmu et al. 1995, Kääb, Ito et al. 2000, Thibault, Robin Poole et al. 2002, Fehrenbacher, Steck et al. 2003), which is a structurally very similar tissue to growth plate, have shown that static and dynamic loading regulates protein synthesis differently. Since growth plate mechanical properties are also related to protein contents, we

hypothesized that changes in the growth plate biomechanical responses are related to changes in protein syntheses. Therefore, the objective of this study was to evaluate the effect of static vs. dynamic compression as well as of dynamic compression parameters (frequency and amplitude of loading) on the main structural growth plate proteins (aggrecan, type II collagen and type X collagen). Immunohistochemical signal reaction strengths were evaluated on images of the tissue revealed by DAB substrate for the three of the main structural proteins. Moreover, to quantify the overall content of proteoglycan and collagen of the tissue in different zones, DMMB assay and Hydroxyproline assay were performed for each zone. Finally, to link changes in protein expressions and growth plate biomechanical responses, a fibril reinforced poroelastic model was used to analytically extract the matrix modulus, the fibril modulus and the permeability of the tissue (Korhonen, Laasanen et al. 2003).

5.4 Methods

5.4.1 Animal Model and Mechanical Modulation

Growth plate samples were extracted from 4-week-old swine distal ulnae under sterile conditions. The upper and lower surfaces of explants were trimmed using a vibratome (Vibratome1500 Sectioning System) to obtain two parallel surfaces. The samples were randomly distributed among the 6 groups of experiment: baseline ($n=1 \times 10$), control ($n=1 \times 10$), static ($n=1 \times 10$), and 3 dynamic groups ($n=3 \times 10$). Samples of all groups except baseline were placed in the chambers of a bioreactor (Tissue Growth Technology, Instron, Norwood, USA) and were cultured in Dulbecco's modified Eagle's medium (DMEM) enriched with 20% heat deactivated Fetal Bovine Serum (FBS) and 1% antibiotics (penicillin and streptomycin) at 37 °C in 95% humidity and 5% CO₂ for 48h hours. For the mechanically modulated groups (static and dynamic), the compression was performed using the parameters indicated in Table 5-1 for 12 hours with the method described in our previous study (Kaviani, Londono et al. 2015).

Table 5-1 Experimental groups and mechanical modulation parameters

Groups	Stress (MPa)	Amplitude (%)	Frequency (Hz)
Baseline	Fresh samples		
Control	Cultured for 48 hours		
Static	0.1	-	-
Dynamic			
Dyn1	0.1	30	0.1
Dyn2	0.1	30	1.0
Dyn3	0.1	100	0.1

After mechanical modulation and/or culturing period (or right after dissection for the baseline group), the explants were divided into four parts. Two parts were used in our previous studies for viability assessment (Kaviani, Londono et al. 2015), mechanical characterization and histomorphological analysis (Kaviani, Londono et al. 2015). One part was fast frozen and preserved for biochemical content evaluation. The last part was fixed in 4% Paraformaldehyde (Sigma-Aldrich), decalcified in 10% EDTA, dehydrated in sequences of alcohol and xylene and finally embedded in paraffin for immunohistochemistry.

5.4.2 Determination of Sulfated Glycosaminoglycan and Hydroxyproline Contents

Each frozen part of explant was further divided into its three zones of reserve, proliferative and hypertrophic using a Microtome Cryostat HM 500 O (GMI, Minnesota, USA) based on the individual histomorphological measurements performed in our previous study (Kaviani, Londono et al. 2015). Each zone of each sample was digested overnight at 60° C with papain enzyme (Sigma-Aldrich GmbH, Steinheim, Germany) at pH 6.0. For determination of sulfated glycosaminoglycan content, the Dimethylmethylene Blue Assay (DMB assay) was performed on Papain digested samples based on the protocol suggested by Hoemann et al., 2004 (Hoemann 2004). Different dilutions of chondroitin sulfate A sodium salt from shark (C4384, Sigma, Montreal, Canada) were used to produce the standard curve. The collagen content was estimated using the hydroxyproline (HYP) assay performed on papain digested samples, which were further acid-hydrolyzed at 110° C for 12 hours. Different dilutions of hydroxyproline solution (Sigma-Aldrich GmbH, Steinheim, Germany) were used to produce the standard curve. The evaluated glycosaminoglycan and collagen contents were normalized to the tissue wet weight.

5.4.3 Immunohistochemistry

The paraffin embedded samples were cut to obtain 5 μ m sections using a rotatory microtome (Leica RM 2145, Wetzlar, Germany). Three antibodies were tested on each explant in triplicate on sections cut at three different depths in the tissue. The protocol used was similar to the one used in the study by Sergerie et al (2011) (Sergerie, Parent et al. 2011). All sections were deparaffinized and rehydrated using xylene and sequences of alcohol, respectively. The sections (for aggrecan and type II collagen antibodies) were incubated for 90 min at 37 °C with chondroitinase ABC so that the epitopes recognized by each monoclonal antibody be exposed. Antigen retrieval was performed by heating the sections in 10mM pH 10.0 TRIS buffer (for aggrecan and type X collagen) or in 10 mM pH 6.0 citrate buffer (for type II collagen) for 20 min at 60 °C.

The sections were then digested in hyaluronidase (2mg/ml; Sigma-Aldrich, St. Louis, MO) for 30 min (for aggrecan and type II collagen antibodies) or 60 min (for type x collagen) at 37 °C. Endogenous peroxidase blocking was performed using hydrogen peroxide in PBS (for aggrecan and type II collagen antibodies) or hydrogen peroxide in methanol (for type x collagen) for 30 min at room temperature. In order to prevent non-specific reactions samples were incubated with 1.5% goat normal serum in PBS (for aggrecan and type II collagen) or 15% non-fat dry milk in TBS buffer (for type X collagen) for 60 min at room temperature. The sections were incubated overnight at 4 °C in presence of primary antibody in a humid chamber, polyclonal rabbit anti-mouse anti-aggrecan (1/50; Chemicon, Temecula, CA), monoclonal mouse anti-chicken anti-type II collagen (1/50; DSHB, Iowa, USA) or monoclonal mouse anti-porcine anti-type X collagen (1/50; Sigma-Aldrich). The next day, the sections were washed and were incubated with secondary antibody: biotinylated anti-rabbit or anti-mouse IgG secondary antibodies (1/200; Vector Laboratories, Burlingame, CA) for 60 min at room temperature. The avidin-biotin complex method (Vectastain ABC kit; Vector Laboratories) was used for detection of antibodies and the reactions were developed using diaminobenzidine substrate (DAB, Vector Laboratories). Finally, the counterstaining was performed using Harris modified hematoxylin solution (Fisher Scientific, Ottawa, ON, Canada) and sections were mounted with Permount (Fisher Scientific). In order to test the specificity of reactions, negative control samples were prepared by eliminating the primary antibody from the procedure.

5.4.4 Imaging and Quantitative Analysis

In order to compare the results from different samples together, all the conditions for preparing the samples (including fixation time, dehydration and paraffin embedding), immunohistochemistry procedure (duration of incubation with different enzymes and antibodies and their concentration) and imaging (parameters used for taking images) were controlled and kept the same for all samples. The images were taken using a Leica DMR microscope equipped with a Retiga Qimaging Camera using the same sets of parameters for each dataset. Quantification of the reaction signal intensity was performed on the resulting RGB images using a custom developed image processing toolbox. The reaction sections were segmented using a K-Mean clustering algorithm and all the other parts were neglected. The resulting segmented RGB image was converted to an indexed image by calculating the mean square root of its RGB value. The reaction signal intensity over a region was calculated by averaging the indexed values over that region. This quantification was performed for each zone of growth plate and for each antibody in triplicate.

The performance of the algorithm was validated on artificially produced images. An example of the algorithm applied on one immunohistochemical image is presented in Figure 5-1.

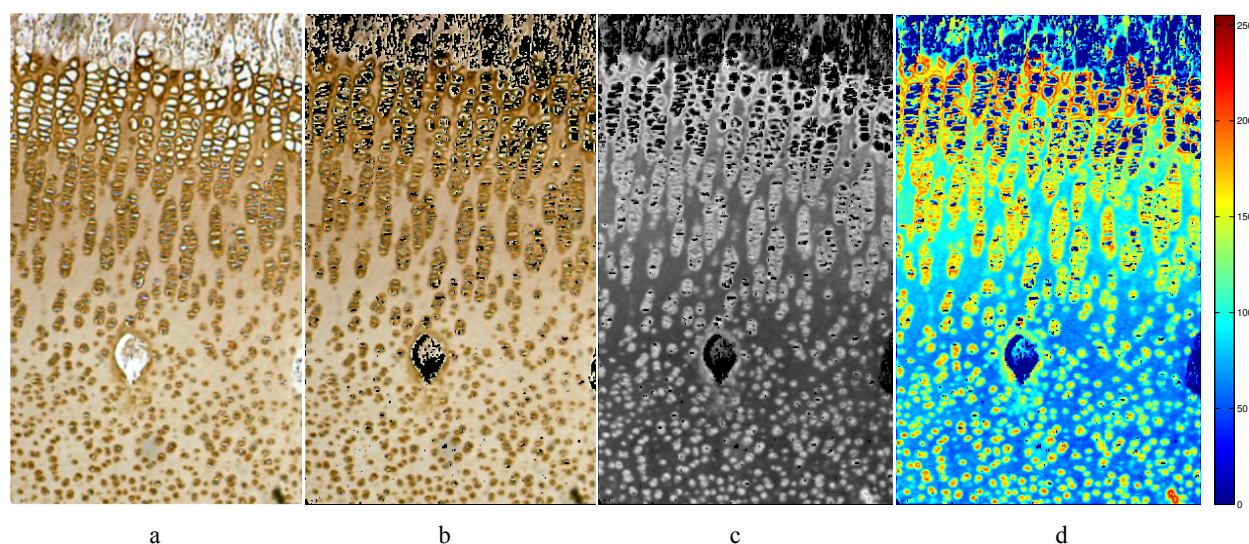


Figure 5-1 Quantification of reaction signal intensity: a) original image; b) segmented parts containing reactions; c & d) indexed images

5.4.5 Mechanical Test and Curve Fitting

In order to find the relationship between growth plate protein expressions and biomechanical responses, a semi-confined stress relaxation test was performed using a custom developed mechanical testing machine on one section of each explant (Kaviani, Londono et al. 2015). Following the first contact between the semi-cylindrical explants and platens, a pre strain of 5% was applied to the tissue. After relaxation, the explants were loaded with 5% of strain and the stress data was collected using a load cell until relaxation. The relaxation criterion was set to $1\text{E-}6$ N/s as explained in our previous study (Kaviani, Londono et al. 2015). During the whole stress relaxation procedure, samples were bathed in DMEM at room temperature. Platen displacements were controlled by a custom designed software (Lab View, National Instruments, Inc., USA) and the force data was recorded using a load cell with resolution of 0.026 N and range of 0 to 40 N.

The MACH-1 analysis software (Biomomentum Inc., Laval, QC, Canada) was used to fit the fibril-network reinforced biphasic model to the stress relaxation curves and calculate the fibril modulus (E_f), the matrix equilibrium modulus (E_m) and axial permeability (k) of explants.

5.4.6 Statistics

Results are reported as means \pm standard deviations for biochemical content, reaction signal intensity and biomechanical parameters. One-way analysis of variance (ANOVA) and Tukey's post hoc comparison were used to determine significant differences between groups. P-values less than 0.01 were considered as significant. Statistical analyses were performed using the MATLAB statistical toolbox (2015a, The Math-Works, Inc., Natick, MA).

5.5 Results

5.5.1 Proteoglycan and Collagen Content

Results of biochemical content analyses for proteoglycan and collagens are summarized in

Table 5-2. Figure 5-2 depicts the average content of collagen and proteoglycan for each zone and their confidence intervals. For all experimental groups, the highest proteoglycan content was found in the proliferative zone and the lowest in the hypertrophic or reserve zone. In order to isolate the culture effect, the control group was compared with the baselines. No significant

differences were observed between the proteoglycan and collagen content of the two groups. In order to isolate the effect of static vs. dynamic loading, static and Dyn1 groups were compared with controls. Compared to the controls, the proteoglycan content of the proliferative zone of static group was significantly reduced to 36.00 ± 15.51 $\mu\text{g/wet weight}$ while the proteoglycan content of the other zones and the three zones of dyn1 group remained unchanged. Also, the collagen content of both static and Dyn1 group did not have a significant difference compared to the control group. In order to evaluate the effects of frequency and amplitude of loading, Dyn2 and Dyn3 groups were compared with Dyn1. No significant differences were observed in the proteoglycan and collagen content of the two groups with respect to Dyn1.

Table 5-2 Proteoglycan and collagen contents of growth plate explants for reserve (R), proliferative (P) and Hypertrophic (H) zones. Significant differences with respect to the control group are marked with * ($p < 0.01$).

Groups	Proteoglycan ($\mu\text{g/wet weight}$)			Collagen ($\mu\text{g/wet weight}$)		
	H	P	R	H	P	R
Baseline	36.37 ± 11.21	67.21 ± 16.64	35.84 ± 14.03	65.96 ± 9.23	32.92 ± 10.27	92.92 ± 17.64
Control	40.67 ± 20.79	60.06 ± 20.25	32.46 ± 11.12	60.81 ± 6.97	45.75 ± 12.59	84.34 ± 19.90
Static	23.84 ± 9.11	$36.00 \pm 15.51^*$	29.08 ± 14.24	74.74 ± 9.73	55.62 ± 17.61	90.94 ± 25.71
Dynamic						
Dyn1	30.62 ± 9.36	56.32 ± 14.62	30.1 ± 8.64	58.75 ± 10.90	50.11 ± 13.63	76.10 ± 18.10
Dyn2	36.66 ± 8.36	56.21 ± 18.64	32.72 ± 11.18	54.86 ± 5.74	45.12 ± 49.00	78.91 ± 16.22
Dyn3	35.32 ± 11.54	57.87 ± 22.81	33.06 ± 8.93	53.10 ± 5.92	39.35 ± 18.39	80.34 ± 17.21

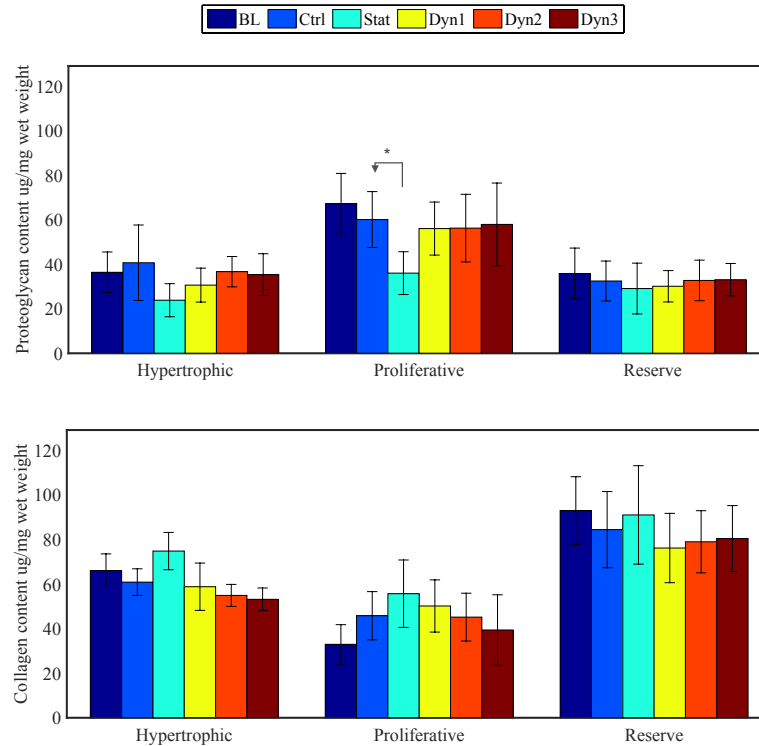


Figure 5-2 Proteoglycan and collagen contents of the reserve, proliferative and hypertrophic zones for the six experimental groups. Significant differences with respect to the control group are marked with * ($p < 0.01$).

5.5.2 Immunohistochemistry Reaction Signal Intensity

Representative images from immunohistochemical analyses for the three antibodies (aggrecan, type II collagen and type X collagen) along with their average reaction signal intensities are presented in Figure 5-3-5, respectively. Aggrecan expression remained unchanged in response to culturing in the three zones of growth plate. Static loading reduced the expression of aggrecan in the proliferative zone and early hypertrophic zones of explants while dynamic loading did not alter the expression of aggrecan with respect to controls (Figure 5-3). Type II collagen expression was not affected neither by culturing nor mechanical modulation (Figure 5-4).

Type X Collagen was exclusively expressed in the hypertrophic zone for all experimental groups (Figure 5-5). The average type X collagen reaction signal intensity for each group of experiment is presented in Figure 5-5. The reaction signal intensity in the controls remained unchanged with respect to the baselines. However, the reaction signal intensity was reduced in all the

mechanically modulated groups. Compared to the Dyn 1 group, static and Dyn 3 groups had lower reaction signal intensities.

5.5.3 Mechanical Properties

No significant differences were observed between collagen fibril moduli of the different groups. All moduli were in the range of 15.5 to 19 MPa (Figure 5-6). The matrix moduli of all the cultured groups were decreased with respect to the baseline group (Figure 5-6). In the baseline group, the average matrix modulus was 1.39 MPa while it was decreased to 0.45 MPa in the culture control group. The permeability was increased in the culture control group with respect to the baseline group. Moreover, the permeability of static group was significantly increased compared to the culture control group (Figure 5-6).

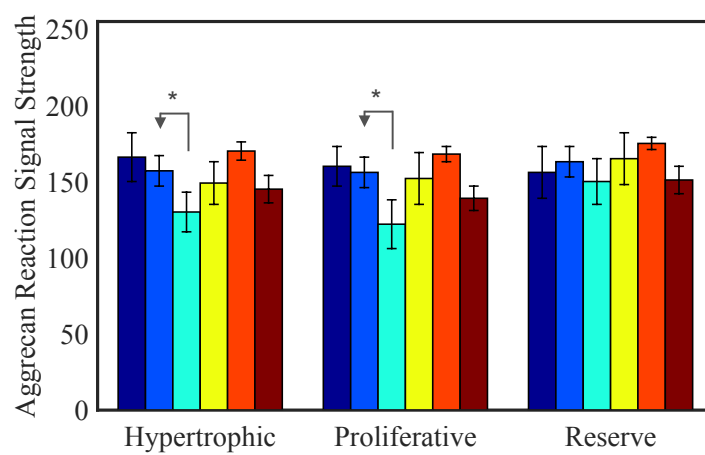
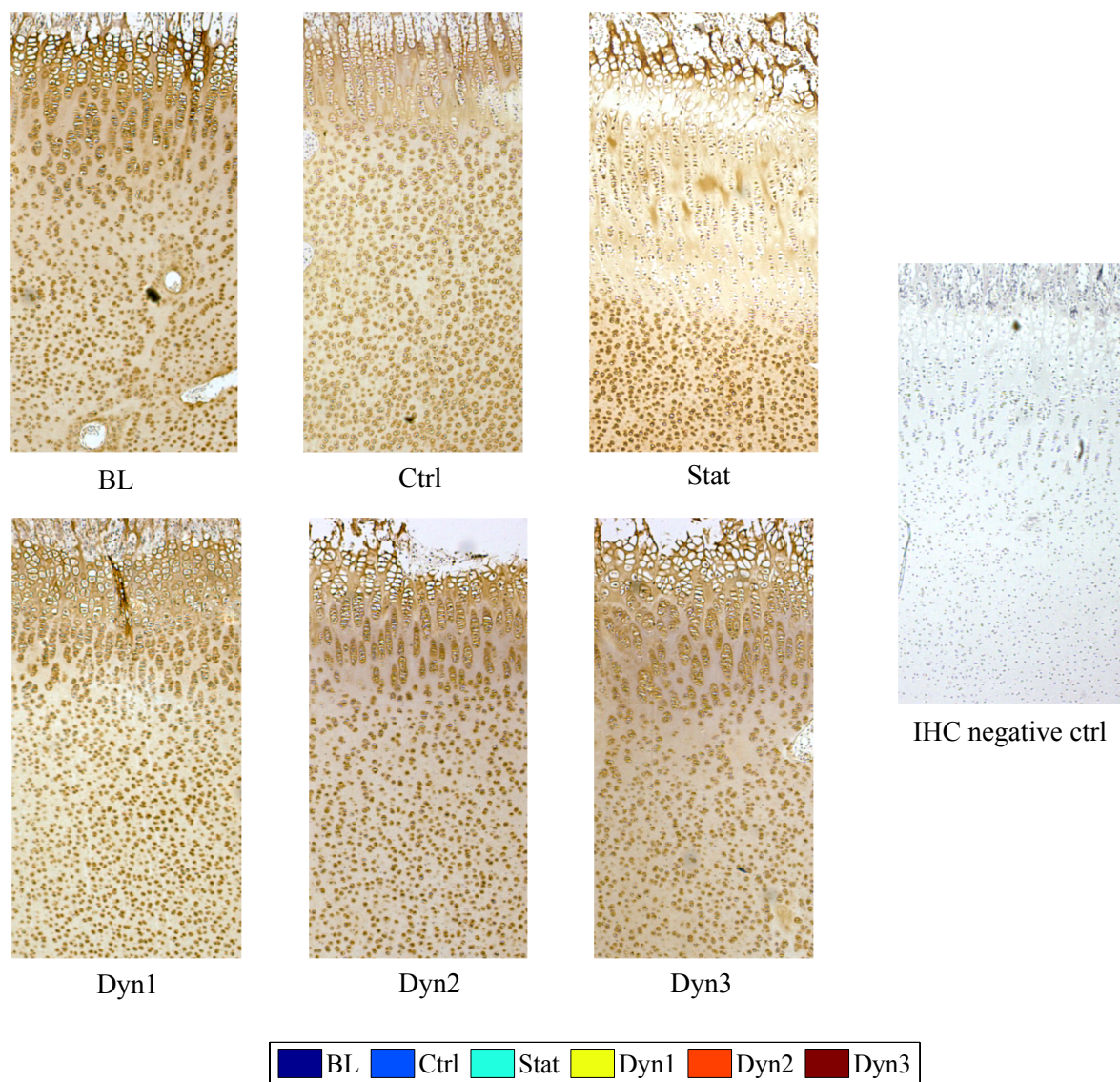


Figure 5-3 A) Representative aggrecan immunohistochemical slides B) Average aggrecan reaction signal intensity

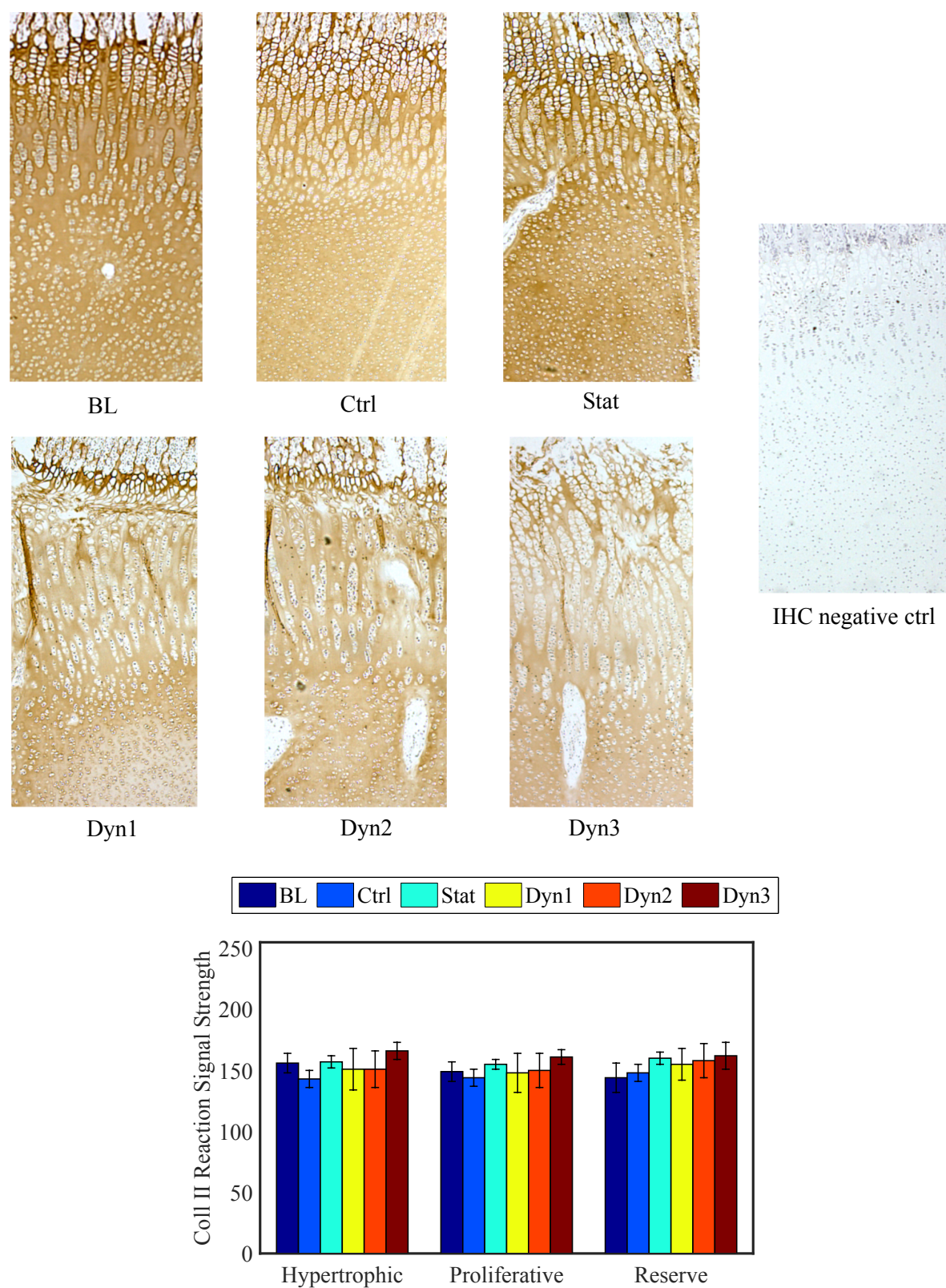


Figure 5-4 A) Representative type II collagen immunohistochemical slides B) Average type II collagen reaction signal intensity

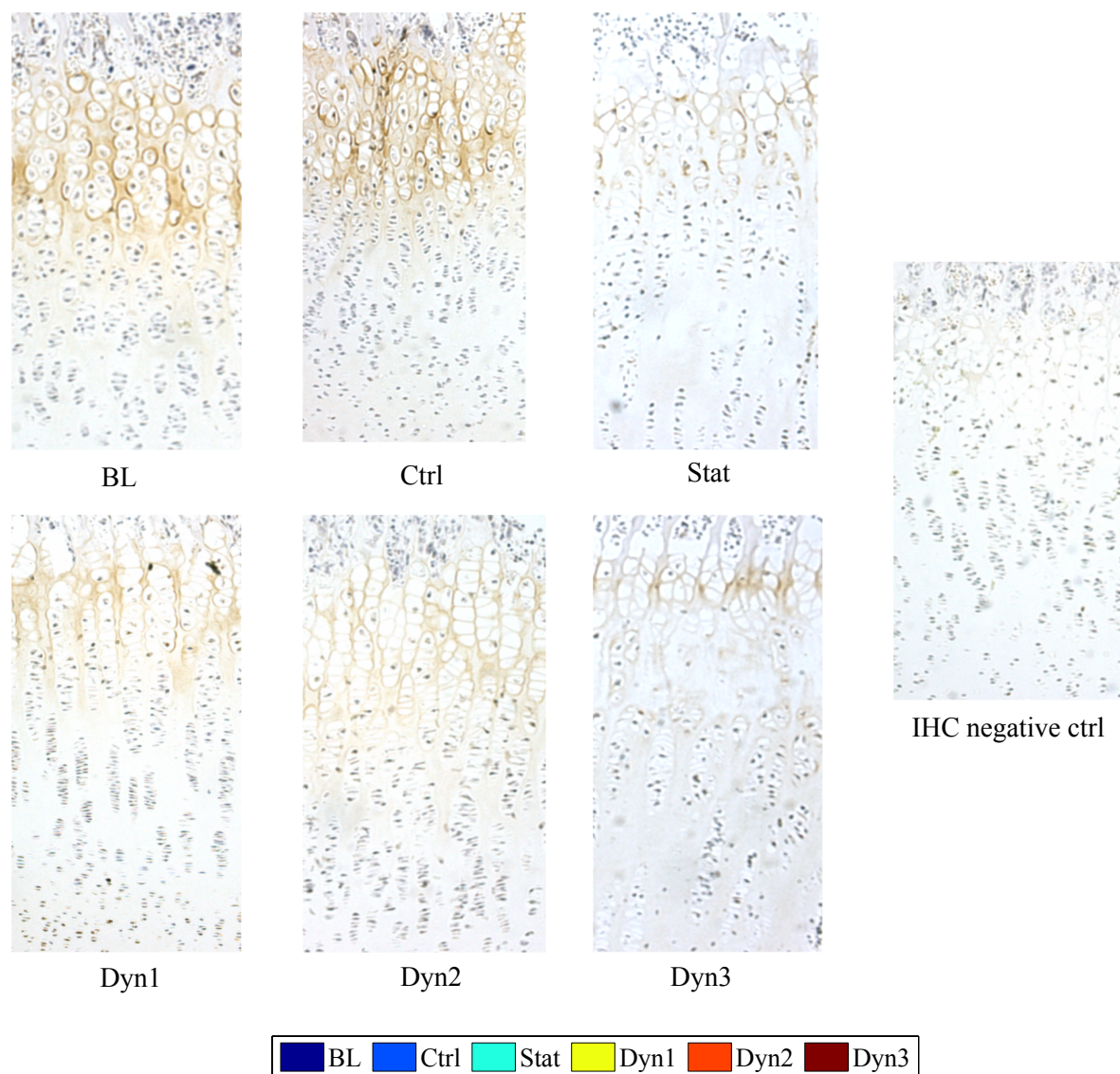


Figure 5-5 Representative type X collagen immunohistochemical slides B) Average type X collagen reaction signal intensity

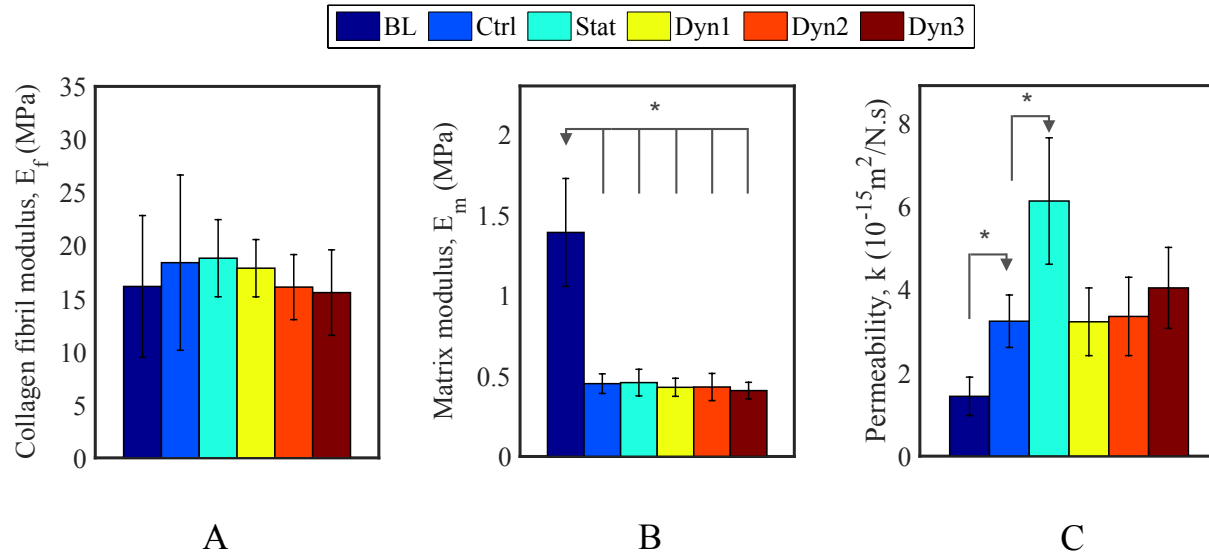


Figure 5-6 Mechanical properties of growth plate explants from the different groups

5.6 Discussion

Static modulation affects the extracellular matrix protein content and mechanical properties of growth plate. Compared to the culture control group, the proteoglycan content of the static group was reduced by 40% in the proliferative zone. Also, based on the immunohistochemistry staining, the expression of aggrecan, one of the main proteoglycan in the growth plate extracellular matrix, was also reduced by 21% in the proliferative zone and by 17% in the hypertrophic zone mostly located at early hypertrophic area. In the dynamically modulated samples, aggrecan expression and proteoglycan content were not changed with respect to controls. These results are consistent with a previous study from our lab (Sergerie, Parent et al. 2011), where a loss of aggrecan was noted in response to static modulation while aggrecan expression was increased in dynamically modulated samples. However, that previous study lacked a reliable comparative system and the immunohistochemistry images were compared by visual inspection, which might explain the differences between results of the two studies for dynamic modulation.

Similar effects have been observed in other cartilaginous tissues such as intervertebral disc (Lotz, Hsieh et al. 2002) and articular cartilage (Bachrach, Valhmu et al. 1995, Ragan, Badger et al. 1999). In both tissues and for the compressive modulation within a similar range as in our study, aggrecan and proteoglycan syntheses were reduced in response to static loading and remained unchanged or was increased in response to dynamic loading (Steinmeyer, Knue et al. 1999, Hee, Zhang et al. 2010). For compressive modulation higher than these values, dynamic loading also had detrimental effects on proteoglycan synthesis (Torzilli, Grigiene et al. 1997).

The loss of proteoglycans occurs through three different mechanisms: increased catabolism, increased porosity of extracellular matrix caused by damages in collagen network or increased deformation-induced convective fluid flow (Sauerland, Raiss et al. 2003). In our study, changes in aggrecan expression is not a result of increased catabolism caused by elevated aggrecanase activity as our group previously showed that ADAMTS-4 and -5 levels do not change in response to static loading (Cancel, Grimard et al. 2009).

Type X collagen is exclusively found in the hypertrophic zone of growth plate and is a marker of chondrocyte maturation and endochondral ossification (O'Keefe, Puzas et al. 1994). The synthesis of type X collagen was reduced in the hypertrophic zone of static explants compared to controls and base dynamics (Dyn1). This correlates with results from a previous similar study in our lab (Sergerie, Parent et al. 2011). Moreover, increasing the amplitude of loading from 30% to 100% reduced type X collagen expression in Dyn 3 group.

Type II collagen, which is found all over the growth plate extracellular matrix, was not affected by the mechanical modulation parameters used in this study. However, our results are not consistent with our previous study on growth plate (Sergerie, Parent et al. 2011) or studies on articular cartilage (Ragan, Badger et al. 1999, Thibault, Robin Poole et al. 2002, Ackermann and Steinmeyer 2005), where type II collagen fibril network was reported to be disrupted by mechanical loading in a dose dependent way.

Changes in matrix modulus and permeability of static explants could be associated with changes in their proteoglycan content.

The permeability of static explants was increased compared to culture control and dynamic groups. This increment of permeability could be directly correlated with the observed decreased proteoglycan content and aggrecan expression in the proliferative zone and type X collagen

reduction in the hypertrophic zone. Based on many studies on growth plate and articular cartilage, proteoglycans are one of the main determinants of the mechanical properties of extracellular matrix in compression (Amini, Mortazavi et al. 2013). Hence, the decrease in proteoglycan content of the proliferative zone and lower expression of aggrecan might partly explain the increase in the higher mechanical strain observed this zone in our previous study (Kaviani, Londono et al. 2015). Changes in the mechanical properties (permeability) of the tissue in response to mechanical modulation have also been reported in articular cartilage (Thibault, Robin Poole et al. 2002). However, in articular cartilage, the increased permeability has shown to be a consequence of the collagen network breakdown in dynamically loaded samples.

Dynamic loading parameters do not impact the growth plate composition and mechanical properties. Changing the modulation frequency from 0.1 Hz to 1.0 Hz, or changing the amplitude of dynamic loading from 30% to 100% did not affect the studied protein expressions, contents or their biomechanical responses. These results correlate with previous studies on the effects of frequency or amplitude of dynamic modulation on growth plate responses, where changing the dynamic loading parameters did not affect growth plate *in vivo* (Ménard, Grimard et al. 2014), *in vitro* (Kaviani, Londono et al. 2015) histomorphology, *in vivo* growth rates (Ménard, Grimard et al. 2014), and *in vitro* compressive strain patterns (Kaviani, Londono et al. 2015). However, these results do not corroborate with results of our previous study on chondrocytes viability (Kaviani, Londono et al. 2015), where a decreased viability was observed in the hypertrophic and proliferative zones when increasing frequency and amplitude, respectively. Similarly, studies on articular cartilage have shown that synthesis of type II collagen is not affected by frequency (Wolf, Ackermann et al. 2007).

Culturing changes the mechanical properties without affecting protein composition of growth plate. In order to differentiate the effects of culturing with the effects of mechanical modulation, the culture group was compared with the baselines. Culturing did not affect the protein expression and protein content of growth plate compared to the baselines. However, the permeability of the explants was increased and their matrix modulus was decreased in response to

culturing while the fibril modulus remained unchanged. These changes could result from a lack of microcirculation.

Overall, this study provides insights on the effect of static vs. dynamic compression as well as dynamic parameters on growth plate protein synthesis and biomechanical responses. Results suggest that static mechanical modulation induces more changes in the extracellular matrix protein expression of growth plate compared to dynamic modulation. Indeed, static compression modified the extracellular matrix composition, namely a decrease in aggrecan and type X collagen, which was translated in an increased permeability of the tissue. Conversely, dynamic compression preserved the growth plate extracellular matrix and biomechanical properties. Although these results cannot be directly extrapolated to an *in vivo* context, they are useful in finding the optimal and less damaging parameters for development of new fusionless treatments based on the mechanical modulation of bone growth.

5.7 Acknowledgements

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CHAPTER 6 GENERAL DISCUSSION

The present study aimed at characterizing the effects of static vs. dynamic and dynamic loading parameters on biomechanical responses, histomorphometry and protein synthesis of growth plate explants. This discussion is a summary and a complement to the discussions presented in Chapter 3, Chapter 4 and Chapter 5.

6.1 Growth plate chondrocyte viability

Chondrocytes like many other cells respond to their mechanical environment. If the loading condition is detrimental for the tissue, chondrocytes will lose their viability. In order to study the effects of mechanical loading on biomechanical and biological responses, first we should make sure that an acceptable number of chondrocytes remain viable in response to mechanical modulation and culturing. Therefore, as a preliminary step in our project, we addressed growth plate chondrocyte viability in response to different loading parameters (Chapter 3).

Our objective was to quantify the viability of chondrocytes in each zone of growth plate in response to modulation with different parameters. In our first study, the effect of type of loading, magnitude, duration, frequency and amplitude of dynamic loading were tested. The mechanical loading parameters and the experimental groups are listed in

Results of this study helped us to identify the effects of different loading parameters on the viability of chondrocytes from different zones of growth plate as explained in the following:

- Hypertrophic and proliferative zones were shown to be the two most sensitive zones to mechanical loading and culturing as their viability was more affected by them.
- Loading magnitude and duration were the two modulation parameters having the most effects on the viability of growth plate chondrocytes.
- When applied for long durations, static loading was more detrimental for chondrocytes viability than dynamic loading with matched parameters.
- The frequency and amplitude of dynamic loading affected the viability of hypertrophic and proliferative loading.

The viability in this study was assessed by a dual staining procedure using live/dead cell viability kit. The live cells were stained using Calcein AM that label cells based on cytoplasmic esterase activity, which only exists in live cells. Dead cells were stained using Ethidium Homodimer 1, which label cells based on their membrane integrity. Ethidium Homodimer 1 can only enter cells with compromised membrane and attach to their nuclei and produce a red fluorescent signal.

Different loading parameters might trigger different signalling cascades in cells leading to either necrosis or apoptosis. In this study, using the above-mentioned method, only necrosis was investigated.

In our preliminary studies though, we evaluated the effects of several loading conditions on the number of apoptotic chondrocytes using the TUNEL assay. No significant differences were observed, between loaded samples and controls. However, this part of our study lacked a sufficient statistical power to provide conclusive results. More investigations would be required in this area.

6.2 Growth plate mechanical responses

As mentioned before, the mechanical environment of chondrocytes plays an important role in regulating their protein synthesis and, as a result, the growth of long bones and vertebrae. It was previously shown in several studies that, under compression, strain distribution is zone dependent in growth plate (Villemure, Cloutier et al. 2007, Amini, Mortazavi et al. 2013). As mechanical environment changes, the synthetic activity of chondrocytes will be changed and as a result the extracellular matrix mechanical responses, including the strain pattern will be changed. This approach was then chosen to characterize strain patterns of growth plate explants in response to different loading parameters (Chapter 4). Moreover, stress relaxation data were fitted on a fibril reinforced biphasic model in order to calculate permeability, matrix modulus and fibril modulus (Chapter 5).

Results of this study helped us identifying the changes in mechanical force distribution and other mechanical characteristics in growth plate to different mechanical modulation parameters. Key conclusions of the study are listed below:

- Static modulation changed the strain pattern within the growth plate samples when compared to the control and dynamic groups.

- Different dynamic loading parameters did not impact the growth plate strain patterns.
- The permeability of explants was increased both in response to culturing and static modulation.
- Frequency and amplitude of dynamic loading did not affect mechanical responses of growth plate tissue.

6.3 Growth plate histomorphology

Histomorphological parameters both at tissue and cell levels can be indicators of growth rate of bones in *in vivo* study (Wilsman, Leiferman et al. 1996). In our study, since we used an *in vitro* approach, we were not able to directly evaluate the effects of different modulation parameters on bone growth rate. As a result, histomorphological responses were measured as an indirect evaluation of the effects of different loading conditions on bone growth rate. Results of this part of the study helped us evaluating the possible changes in growth rate of bones in response to different compressive modulation parameters (Chapter 4). Key conclusions of the study are presented below:

- Thicknesses of the three zones of growth plate, which can be an indicator of growth rate, were not affected by mechanical modulation parameters.
- The columnar arrangement of chondrocytes in the hypertrophic and proliferative zones were lost in response to static loading while in dynamic groups, the columns were only slightly deviated from the direction of growth.
- Frequency and amplitude of dynamic loading did not affect histomorphological responses of growth plate explants.

In this study, histomorphological parameters were measured using a semi automatic image processing procedure in two dimensions. Data presented in (Chapter 4) only encompass the tissue histomorphological parameters. During preliminary studies, the effects of different modulation parameters on cellular histomorphology were also studied and no significant differences were observed on the height of hypertrophic chondrocytes and number of proliferative chondrocytes. This part of our study was not presented because of insufficient statistical power.

Moreover, since the histomorphological changes are occurring in 3 dimensions, performing a 3 dimensional histomorphometry study would provide more realistic evaluation of the effects of

mechanical loading on histomorphological parameters and should be performed to complete this study in the future.

6.4 Growth plate protein content and protein expression

As mentioned in section 1.1.2.1, growth plate extracellular matrix is principally composed of water, proteoglycan and collagen. Aggrecan is the major proteoglycan and type II collagen is the major collagen in the extracellular matrix of growth plate. Also, type X collagen, which is exclusively found in the hypertrophic zone, is one of the main components of growth plate. Since these components give growth plate its special mechanical characteristics, any changes in the mechanical responses followed by mechanical modulation should be accompanied by changes in the expression of one or several of these proteins. That is the reason behind our fourth objective in this study where we evaluated the effects of different mechanical modulation parameters on GAG and collagen contents as well as on aggrecan, type II collagen and type X collagen expressions (Chapter 5).

Results of this study helped identifying changes in protein expressions in growth plate in response to different mechanical modulation parameters, which are expected to be associated to changes in the mechanical responses. Key conclusions of the study are listed below:

- In response to static modulation, GAG content and aggrecan expression was reduced in the proliferative zone.
- Type X collagen expression was decreased in response to mechanical modulation whether it was static or dynamic.
- Frequency and amplitude of dynamic modulation did not affect proteoglycan and collagen syntheses in growth plate.

Proteoglycan content of the growth plate explants was estimated by measuring the GAG content using DMMB colorimetric assay. Results of this part are consistent with the results of our histological study using Safranin O staining, which stains proteoglycans and GAGs (Schmitz, Lavery et al. 2010). Among the sections stained with Safranin O, all the cultured samples showed a less intense staining in comparison to the baselines. Moreover, static samples' staining was even less intense in the hypertrophic and proliferative zones than the other groups. Figure

6-1 shows, for each group, a representative growth plate section stained with Safranin O and counter stained with fast green stains.

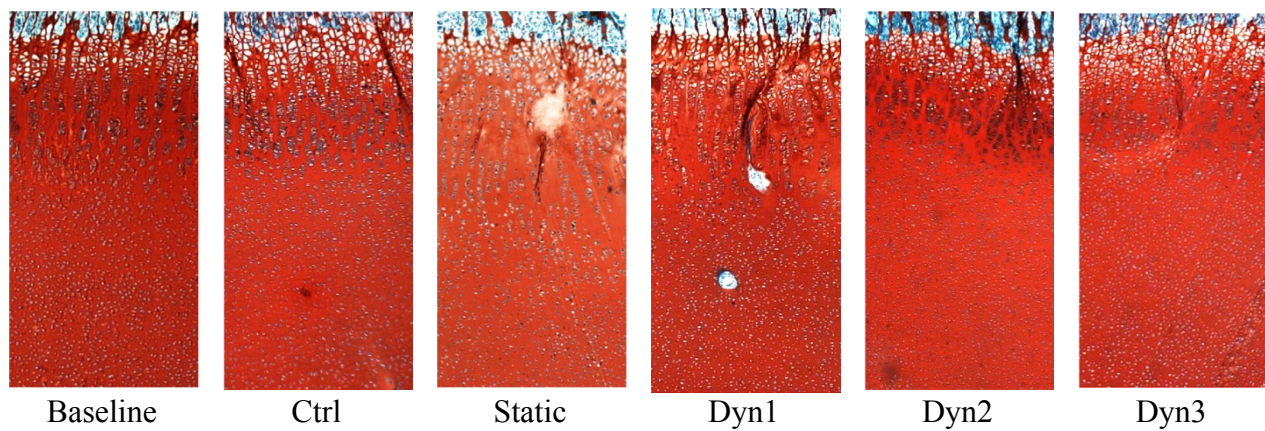


Figure 6-1 Sections stained with Safranin O

Moreover, in order to study the effects of mechanical loading on degrading enzymes, the effects of different modulation protocols on MMP 13 enzyme expression was also evaluated using immunohistochemistry. This enzyme was chosen because it has a role in degradation of both aggrecan and collagen. However, because all the sections had a strong background we did not present these results in Chapter 5. Here, we have calculated the reaction signal intensity for MMP 13; as the background was similar for all samples, its effect will be similar on reaction signal intensity. Therefore, we could still compare different groups together. Figure 6-2 presents the reaction signal intensity for different zones of growth plate for MMP 13. No significant differences were observed on the expression of MMP 13 between the six different groups of experiment.

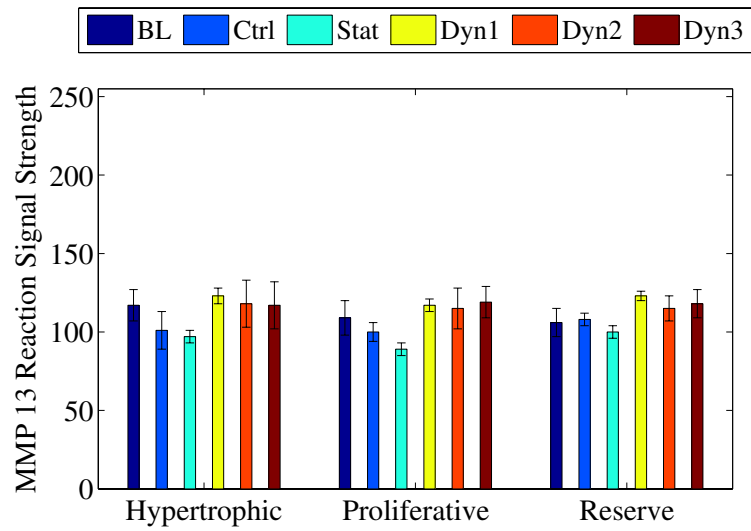
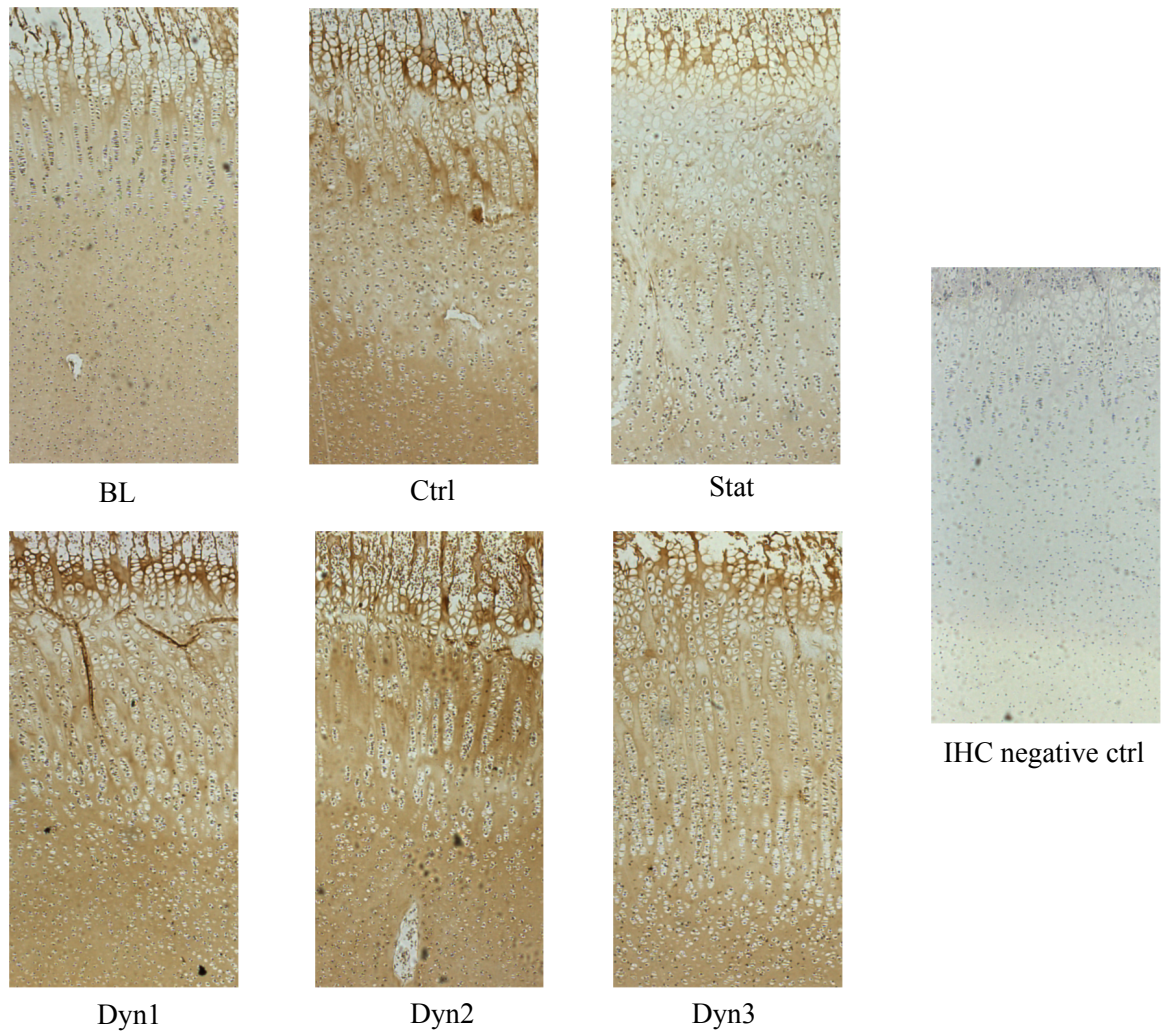


Figure 6-2 MMP 13 reaction signal intensity in the three zones of growth plate

6.5 Global discussion and limits of the project

At the chondrocyte level, chondrocytes viability was shown to be modulated with different modulation parameters. Loading magnitude and duration had the most destructive effects on the chondrocytes viability in all the three zones of growth plate both in static and dynamic loading. Loading duration, however, had a more pronounced effect in statically loaded explants. As a result, in the long term, static loading had more detrimental effects. Increasing the frequency, affected the chondrocytes viability in the hypertrophic zone while increasing loading amplitude affected the viability in the proliferative zone. This suggests that frequency and amplitude might trigger different signalling cascades, which lead to cells death in different parts of the tissue. The underlying mechanism for this needs to be further investigated.

At the mechanical level, static loading changed the strain pattern of the tissue with respect to the culture controls. The strain pattern shows how the forces are transmitted throughout the growth plate and helps us in finding the areas, which undergo a large compression following mechanical modulation. Moreover, the permeability of the tissue was increased in response to both culturing and static modulation. The increased permeability can explain the decreased modulus of elasticity for all cultured groups. Moreover, it was shown in studies on articular cartilage, that water content is increased by increment of permeability (Armstrong and Mow 1982, Mow, Holmes et al. 1984). Therefore, it can be assumed that cultured explants had more water content with respect to the baselines and that statically modulated samples had an increased water content compared to the other cultured groups. The fibril modulus, which mostly shows the effect of collagen fibers resistance to loading (Korhonen, Laasanen et al. 2003), was not affected by any type of loading while matrix modulus showing mostly the effects of proteoglycans (Korhonen, Laasanen et al. 2003) was decreased in response to culturing. Therefore, changes in the mechanical responses of static samples could be associated with this decrement in the proteoglycan content and consequently an increased water content of the tissue.

At the protein level, type II collagen was not modulated by different mechanical parameters while static loading decreased the PG contents and aggrecan expression in the hypertrophic and proliferative zones. The decreased PG content can be associated with the increased deformation

observed in the hypertrophic zone of the static group. Moreover, it was shown in articular cartilage that proteoglycan content has an indirect relationship with permeability of the tissue (Armstrong and Mow 1982, Mow, Holmes et al. 1984). As a result, the increased permeability of static group can also be associated with the decreased PG content. Moreover, the decreased matrix modulus can also be associated with the decreased PG content (Korhonen, Laasanen et al. 2003). Type X collagen expression was decreased in all the mechanically modulated groups, indicating that this protein is very sensitive to its mechanical environment. Its expression was most affected in the static group as it underwent more deformation at the hypertrophic zone.

At the histomorphometry level, it was shown that static modulation disrupts the columnar arrangement of chondrocytes in the hypertrophic and proliferative zones while dynamic loading preserves the columnar arrangement. The loss of columnar arrangement can be an indicator of the loss of extracellular matrix integrity, which can be associated with proteoglycan decrement in the hypertrophic and proliferative zones and type X collagen decrement in the hypertrophic zone. Moreover, since proper organization of chondrocytes in columns is necessary for proper growth (Wilsman, Leiferman et al. 1996), this loss of columnar arrangement could further result in abnormal bone growth.

This study has several technical limits that are explained in the following section. First of all, this study was an *in vitro* study; many factors that are available *in vivo* such as systemic and local regulations, which are modulated in response to mechanical loading, were not intrinsically included in the chosen growth plate model. Thus, direct extrapolation of the results to an *in vivo* context is not possible. However, the advantage of this *in vitro* study was that, since factors, such as systemic hormonal and local regulations, were not involved, we could isolate the effects of mechanical parameters in a more controlled way. Secondly, this study was performed with variation between two values for each parameter (magnitude, duration, frequency and amplitude of dynamic loading). To provide a more comprehensive conclusion, the effects of more parameters should be tested. Moreover, the study was limited to one animal model (porcine) at one developmental stage (4 week old). It would be interesting to verify if the same results would be obtained in different species at different developmental stages. The study was performed on healthy animal models; the force transduction may be different in pathologic samples and as a result mechanical modulation could have different effects. At viability level, only one type of test was used for evaluating the number of live and dead cells, which was based on the permeability

of cells. Different parameters might have different effects on cells and as a result trigger different pathways for cells death. It would be interesting to investigate the effect of mechanical modulation on mechanotransduction pathways such as stretch-activated ion channels (SAC), the hyaluronan receptor CD44, annexin V and integrin receptors. At the mechanical characterization level, only one stress relaxation protocol was performed. It would be interesting to study the mechanical characteristics using additional tests, namely dynamic test protocols. At the biological level, the statistical differences could not be distinguished due to high standard deviations, but a general trend could be observed.

CHAPTER 7 CONCLUSION AND RECOMMENDATIONS

7.1 Conclusion

The main objective of this thesis was to characterize the mechanical and biological responses of growth plate explants to different sets of compressive modulation parameters and to find the relationships between changes in biomechanical responses, chondrocytes viability, histomorphology and protein synthesis.

At the first step, in order to implement a culture system suitable for evaluation of mechanical responses, the viability of chondrocytes was evaluated in response to different modulation parameters. Afterward, mechanical characterization was performed for samples that showed an acceptable viability using a combination of imaging, stress relaxation test and digital image correlation. Then, the protein synthetic responses were characterized using semi quantitative immunohistochemistry method and the biochemical content was calculated using colorimetric assays. Finally, a study was performed to find the relationships between these responses.

First of all, viability of chondrocytes was shown to be dependent on the magnitude, duration, frequency and amplitude of loading. Both static and dynamic loading with low magnitude and low duration had a similar effect on viability. As a result, the first research hypothesis stating that *“Static and dynamic modulation trigger different effects on chondrocyte viability, and static modulation will be more detrimental for cellular viability”* was not confirmed. It is now clear that static and dynamic loading with low magnitude and low durations of loading have the same effects on viability of chondrocytes.

Secondly, it was shown that static and dynamic loadings with similar average magnitude result in different strain patterns in the tissue. Moreover, the permeability of samples with static modulation was increased compared to dynamic modulation. Therefore, it can be concluded that dynamic modulation tends to better preserve the biomechanical responses of growth plate. As a result, the second research hypotheses stating that *“Statically and dynamically modulated growth plate explants show different changes in their biomechanical properties and dynamically modulated growth plate explants remain more similar to baseline samples”* was confirmed. It is now confirmed that growth plate strain pattern is modulated by the type of loading in a way that static loading induces more changes to the biomechanical responses of growth plate. Moreover, it

is established that dynamic loading parameters in the range we varied in this study do not change the strain pattern of the tissue.

Furthermore, it was shown that the two dynamic loading parameters (frequency and amplitude) do not affect the mechanical responses of growth plate while frequency slightly affects the hypertrophic zone viability and amplitude slightly affects the proliferative zone viability. Moreover, it was shown that magnitude and duration of loading have the most effects on viability of chondrocytes. Therefore, the third hypothesis stating, “*Among dynamic modulating parameters, frequency causes the most significant changes in growth plate biomechanical properties and chondrocyte viability compared to loading magnitude*” was rejected. It is now clear that magnitude of loading have more modulating effects on biomechanics of growth plate and viability of its chondrocytes.

Finally, it was shown that changes in the strain pattern of the tissue and biomechanical responses of growth plate in static group are associated with loss of proteoglycan and lower expression of aggrecan in the hypertrophic and proliferative zones. Therefore, the final hypotheses of the present study, stating that “*Changes in growth plate biomechanical properties and chondrocyte viability are associated with changes in extracellular matrix integrity and tissue and cell histomorphometry*” was partly established for the proliferative and hypertrophic zones.

In this study, for the first time the effects of mechanical modulation parameters on biomechanical and biological responses of growth plate were investigated using an *in vitro* approach with an animal model with low growth rate, similar to humans. Results of this study are relevant for identifying the optimal and less detrimental loading conditions for the fusionless treatment of scoliosis and other developmental growth deformities.

7.2 Recommendations for future studies

Recommendations for future studies would include the followings:

- Increasing the sample size for protein content studies;
- Evaluating denatured proteins content;
- Evaluating the organization of collagen fibers in response to different mechanical modulation parameters.

- Testing other values of each mechanical modulation parameter;
- Evaluating the effects of mechanical modulation on signal transduction at cell level;
- Evaluating the effects of mechanical modulation parameters on protein expression at mRNA level.
- Confirming the results using other animal models, vertebral growth plates and using an *in vivo* approach;
- Confirming the results using a pathological animal model.

From a clinical point of view, it is of great interest to identify the differential effects of static vs. dynamic modulation on the biomechanical and biological responses of growth plate. Results of this study demonstrate that mechanical characteristics (strain patterns, mechanical properties) and biological responses (cell viability, histomorphometry and protein synthesis) are dependent on the type of loading and static compression modulation would be more detrimental for tissue. This knowledge will be helpful in choosing efficient and non damaging modulation parameters for the development of new fusionless approaches for the treatment of progressive musculoskeletal deformities.

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